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## Box Patent Application

Assistant Commissioner for Patents  
Washington, DC 20231

Presented for filing is a new patent application claiming priority from a provisional patent application of:

Applicant: ALLAN M. MILLER, DOUGLAS A. TRECO, RICHARD F SELDEN  
Title: OPTIMIZED MESSENGER RNA

Enclosed are the following papers, including those required to receive a filing date under 37 CFR §1.53(b):

	<u>Pages</u>
Specification	60
Claims	8
Abstract	1
Signed Declaration	[To Be Filed At A Later Date]
Drawing(s)	34

### Enclosures:

- Sequence Listing (paper copy) 19 pages
- Postcard.

Under 35 USC §119(e)(1), this application claims the benefit of prior U.S. provisional applications 60/130,241, filed April 20, 1999 and 60/102,239, filed September 29, 1998.

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This application is entitled to small entity status. Small entity status established in a previous application is still proper.

Basic filing fee	380.00
Total claims in excess of 20 times \$9.00	387.00
Independent claims in excess of 3 times \$39.00	195.00
Fee for multiple dependent claims	130.00
Total filing fee:	\$ 1,092.00

A check for the filing fee is enclosed. Please apply any other required fees or any credits to deposit account 06-1050, referencing the attorney docket number shown above.

If this application is found to be incomplete, or if a telephone conference would otherwise be helpful, please call the undersigned at 617/542-5070.

Kindly acknowledge receipt of this application by returning the enclosed postcard.

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Respectfully submitted,

 <sup>Reg. No.</sup>  
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Enclosures

APPLICATION  
FOR  
UNITED STATES LETTERS PATENT

**TITLE:** OPTIMIZED MESSENGER RNA

**APPLICANT:** ALLAN M. MILLER, DOUGLAS A. TRECO, RICHARD F  
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Lee Ray  
Anno Ray

## OPTIMIZED MESSENGER RNA

### Cross Reference To Related Applications

This application claims the benefit of prior U.S. provisional application 60/102,239, filed September 29, 1998, and prior U.S. provisional application 60/130, 241, filed April 20, 1999, the contents of which are herein incorporated by reference.

### Field of the Invention

The invention is directed to methods for optimizing the properties of mRNA molecules, optimized mRNA molecules, methods of using optimized mRNA molecules, and compositions which include optimized mRNA molecules.

### Background of the Invention

In Eukaryotes, gene expression is affected, in part, by the stability and structure of the messenger RNA (mRNA) molecule. mRNA stability influences gene expression by affecting the steady-state level of the mRNA; it can affect the rates at which the mRNA disappears following transcriptional repression and accumulates following transcriptional induction. The structure and nucleotide sequence of the mRNA molecule can also influence the efficiency with which these individual mRNA molecules are translated.

The intrinsic stability of a given mRNA molecule is influenced by a number of specific internal sequence elements which can exert a destabilizing effect on the mRNA. These elements may be located in any region of the transcript, and e.g., can be found in the 5' untranslated region (5'UTR), in the coding region and in the 3' untranslated region (3'UTR). It is well established that shortening of the poly(A) tail initiates mRNA decay (Ross, *Trends in Genetics*, 12:171-175, 1996). The poly(A) tract influences cytoplasmic mRNA stability by protecting mRNA from rapid degradation. Adenosine and uridine rich elements (AUREs) in the 3'UTR are also associated with unstable mammalian mRNA's. It has been demonstrated that proteins that bind

to AURE, AURE-binding proteins (AUBPs), can affect mRNA stability. The coding region can also alter the half-life of many RNAs. For example, the coding region can interact with proteins that protect it from endonucleolytic attack. Furthermore, the efficiency with which individual mRNA molecules are translated has a strong influence on the stability of the mRNA molecule (Herrick et al., Mol Cell Biol. 10, 2269-2284, 1990, and Hoekema et al., Mol Cell Biol. 7, 2914-2924, 1987)..

The single-stranded nature of mRNA allows it to adopt secondary and tertiary structure in a sequence-dependent manner through complementary base-pairing. Examples of such structures include RNA hairpins, stem loops and more complex structures such as bifurcations, pseudoknots and triple-helices. These structures influence both mRNA stability, e.g., the stem loop elements in the 3' UTR can serve as a endonuclease cleavage site, and affect translational efficiency.

In addition to the structure of the mRNA, the nucleotide content of the mRNA can also play a role in the efficiency with which the mRNA is translated. For example, mRNA with a high GC content at the 5' untranslated region (UTR) may be translated with low efficiency and a reduced translational effect can reduce message stability. Thus, altering the sequence of a mRNA molecule can ultimately influence mRNA transcript stability, by influencing the translational stability of the message.

Factor VIII and Factor IX are important plasma proteins that participate in the intrinsic pathway of blood coagulation. Their dysfunction or absence in individuals can result in blood coagulation disorders, e.g., a deficiency of Factor VIII or Factor IX results in Hemophilia A or B, respectively. Isolating Factor VIII or Factor IX from blood is difficult, e.g., the isolation of Factor VIII is characterized by low yields, and also has the associated danger of being contaminated with infectious agents such as Hepatitis B virus, Hepatitis C virus or HIV. Recombinant DNA technology provides an alternative method for producing biologically active Factor VIII or Factor IX. While these methods have had some success, improving the yield of Factor VIII or Factor IX is still a challenge.

An approach to increasing protein yield using recombinant DNA technology is to modify the coding sequence of a protein of interest, e.g., Factor VIII or Factor IX, without altering the amino acid sequence of the gene product. This approach involves altering, for example, the native Factor VIII or Factor IX gene sequence such that codons which are not so frequently used

in mammalian cells are replaced with codons which are overrepresented in highly expressed mammalian genes. Seed et al., (WO 98/12207) used this approach with a measure of success. They found that substituting the rare mammalian codons with those frequently used in mammalian cells results in a four fold increase in Factor VIII production from mammalian cells.

### Summary of the Invention

In one aspect, the invention features, a synthetic nucleic acid sequence which encodes a protein, or a portion thereof, wherein at least one non-common codon or less-common codon has been replaced by a common codon, and wherein the synthetic nucleic acid sequence includes a continuous stretch of at least 90 codons all of which are common codons.

The synthetic nucleic acid can direct the synthesis of an optimized messenger mRNA. In a preferred embodiment the continuous stretch of common codons can include: the sequence of a pre-pro-protein; the sequence of a pro-protein; the sequence of a mature protein; the "pre" sequence of a pre-pro-protein; the "pre-pro" sequence of a pre-pro-protein; the "pro" sequence of a pre-pro or a pro-protein; or a portion of any of the aforementioned sequences.

In a preferred embodiment, the synthetic nucleic acid sequence includes a continuous stretch of at least 90, 95, 100, 125, 150, 200, 250, 300 or more codons all of which are common codons.

In another preferred embodiment, the nucleic acid sequence encoding a protein has at least 30, 50, 60, 75, 100, 200 or more non-common or less-common codons replaced with a common codon.

In a preferred embodiment, the number of non-common or less-common codons replaced is less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1.

In a preferred embodiment, the number of non-common or less-common codons remaining is less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1.

In preferred embodiments, the non-common and less-common codons replaced, taken together, are equal or less than 6%, 5%, 4%, 3%, 2%, 1% of the codons in the synthetic nucleic acid sequence.

In preferred embodiments, the non-common and less-common codons remaining, taken together, are equal or less than 6%, 5%, 4%, 3%, 2%, 1% of the codons in the synthetic nucleic acid sequence.

In a preferred embodiment, all of the non-common or less-common codons of the synthetic nucleic acid sequence encoding a protein have been replaced with common codons.

In a preferred embodiment, the synthetic nucleic acid sequence encodes a protein of at least about 90, 95, 100, 105, 110, 120, 130, 150, 200, 500, 700, 1000 or more amino acids in length.

In various preferred embodiments, at least 94%, 95%, 96%, 97%, 98%, 99%, , or all, of the codons in the synthetic nucleic acid sequence are common codons. Preferably, all of the codons in the synthetic nucleic acid sequence are common codons.

In preferred embodiments, the protein is expressed in a eukaryotic cell, e.g., a mammalian cell, e.g., a human cell, and the protein is a mammalian protein, e.g., a human protein.

In another aspect, the invention features, a synthetic nucleic acid sequence which encodes a protein, or a portion thereof, wherein at least one non-common codon or less-common codon has been replaced by a common codon, and wherein the synthetic nucleic acid sequence includes a continuous stretch of common codons, which continuous stretch includes at least 33% or more of the codons in the synthetic nucleic acid sequence.

The synthetic nucleic acid can direct the synthesis of an optimized messenger mRNA. In a preferred embodiment the continuous stretch of common codons can include: the sequence of a pre-pro-protein; the sequence of a pro-protein; the sequence of a mature protein; the "pre" sequence of a pre-pro-protein; the "pre-pro" sequence of a pre-pro-protein; the "pro" sequence of a pre-pro or a pro-protein; or a portion of any of the aforementioned sequences.

In a preferred embodiment, the synthetic nucleic acid sequence includes a continuous stretch of common codons wherein the continuous stretch includes at least 35%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 100% of codons in the synthetic nucleic acid sequence.

In a preferred embodiment, the number of non-common or less-common codons replaced is less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1.

In a preferred embodiment, the number of non-common or less-common codons remaining is less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1.

In preferred embodiments, the non-common and less-common codons replaced, taken together, are equal or less than 6%, 5%, 4%, 3%, 2%, 1% of the codons in the synthetic nucleic acid sequence.

In preferred embodiments, the non-common and less-common codons remaining, taken together, are equal or less than 6%, 5%, 4%, 3%, 2%, 1% of the codons in the synthetic nucleic acid sequence.

In a preferred embodiment, all of the non-common or less-common codons of the synthetic nucleic acid sequence encoding a protein have been replaced with common codons.

In a preferred embodiment, all non-common and less-common codons are replaced with common codons.

In a preferred embodiment, the synthetic nucleic acid sequence encodes a protein of at least about 90, 95, 100, 105, 110, 120, 130, 150, 200, 500, 700, 1000 or more amino acids in length.

In various preferred embodiments, at least 94%, 95%, 96%, 97%, 98%, 99%, or all, of the codons in the synthetic nucleic acid sequence are common codons. Preferably, all of the codons in the synthetic nucleic acid sequence are common codons.

In preferred embodiments, the protein is expressed in a eukaryotic cell, e.g., a mammalian cell, e.g., a human cell, and the protein is a mammalian protein, e.g., a human protein.

In another aspect, the invention features, a synthetic nucleic acid sequence which encodes a protein, or a portion thereof, wherein at least one non-common codon or less-common codon has been replaced by a common codon, and wherein the number of non-common and less-common codons, taken together, is less than  $n/x$ , wherein  $n/x$  is a positive integer,  $n$  is the number of codons in the synthetic nucleic acid sequence and  $x$  is chosen from 2, 4, 6, 10, 15, 20, 50, 150, 250, 500 and 1000. (Fractional values for  $n/x$  are rounded to the next highest of lowest integer, positive values below 0.5 are rounded down and values above 0.5 are rounded up).

The synthetic nucleic acid can direct the synthesis of an optimized messenger mRNA. In a preferred embodiment the continuous stretch of common codons can include: the sequence of a pre-pro-protein; the sequence of a pro-protein; the sequence of a mature protein; the "pre"



sequence of a pre-pro-protein; the "pre-pro" sequence of a pre-pro-protein; the "pro" sequence of a pre-pro or a pro-protein; or a portion of any of the aforementioned sequences.

In a preferred embodiment, the number of codons in the synthetic nucleic acid sequence (n) is at least 50, 60, 70, 80, 90, 100, 120, 150, 200, 350, 400, 500 or more.

In a preferred embodiment, the number of non-common or less-common codons replaced is less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1.

In a preferred embodiment, the number of non-common or less-common codons remaining is less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1.

In preferred embodiments, the non-common and less-common codons replaced, taken together, are equal or less than 6%, 5%, 4%, 3%, 2%, 1% of the codons in the synthetic nucleic acid sequence.

In preferred embodiments, the non-common and less-common codons remaining, taken together, are equal or less than 6%, 5%, 4%, 3%, 2%, 1% of the codons in the synthetic nucleic acid sequence.

In a preferred embodiment, all non-common or less-common codons are replaced with common codons.

In various preferred embodiments, at least 94%, 95%, 96%, 97%, 98%, 99%, or all of the codons in the synthetic nucleic acid sequence are common codons. Preferably, all of the codons in the synthetic nucleic acid sequence are common codons.

In preferred embodiments, the protein is expressed in a eukaryotic cell, e.g., a mammalian cell, e.g., a human cell, and the protein is a mammalian protein, e.g., a human protein.

In another aspect, the invention features, a synthetic nucleic acid sequence which encodes a protein, or a portion thereof, wherein at least one non-common codon or less-common codon has been replaced by a common codon in the sequence that has not been optimized (non-optimized) which encodes the protein, wherein at least 94% or more of the codons in the sequence encoding the protein are common codons and wherein the synthetic nucleic acid sequence encodes a protein of at least about 90, 100 or 120 amino acids in length.

The synthetic nucleic acid can direct the synthesis of an optimized messenger mRNA. In a preferred embodiment the continuous stretch of common codons can include: the sequence of a

pre-pro-protein; the sequence of a pro-protein; the sequence of a mature protein; the "pre" sequence of a pre-pro-protein; the "pre-pro" sequence of a pre-pro-protein; the "pro" sequence of a pre-pro or a pro-protein; or a portion of any of the aforementioned sequences.

In preferred embodiments, at least 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more of non-common or less-common codons in the non-optimized nucleic acid sequence encoding the protein have been replaced by a common codon encoding the same amino acid. Preferably, all non-common or less-common codons are replaced by a common codon encoding the same amino acid as found in the non-optimized sequence.

In a preferred embodiment, the synthetic nucleic acid sequence encodes a protein of at least about 90, 95, 100, 105, 110, 120, 130, 150, 200, 500, 700, 1000 or more amino acids in length.

In other preferred embodiments, at least 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5% of the non-common codons in the non-optimized nucleic acid sequence are replaced with common codons. Preferably, all of the non-common codons are replaced with the common codons.

In other preferred embodiments at least 94%, 95%, 96%, 97%, 98%, 98%, 99%, 99.5% of the less-common codons in the non-optimized nucleic acid sequence are replaced with common codons. Preferably, all of the less-common codons are replaced with the common codons.

In preferred embodiments, at least 94% or more of the non-common and less common codons are replaced with common codons.

In preferred embodiments, the number of codons replaced which are not common codons is equal to or less than 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1.

In preferred embodiments, the number of codons remaining which are not common codons is equal to or less than 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1

In preferred embodiments, the protein is expressed in a eukaryotic cell, e.g., a mammalian cell, e.g., a human cell, and the protein is a mammalian protein, e.g., a human protein.

The synthetic nucleic acid can direct the synthesis of an optimized messenger mRNA. In a preferred embodiment the continuous stretch of common codons can include: the sequence of a pre-pro-protein; the sequence of a pro-protein; the sequence of a mature protein; the "pre"

sequence of a pre-pro-protein; the "pre-pro" sequence of a pre-pro-protein; the "pro" sequence of a pre-pro or a pro-protein; or a portion of any of the aforementioned sequences.

In a preferred embodiment the synthetic nucleic acid sequence is at least 100, 110, 120, 150, 200, 300, 500, 700, 1000 or more base pairs in length.

In another aspect, a synthetic nucleic acid sequence that directs the synthesis of an optimized message which encodes a Factor VIII protein having one or more of the following characteristics:

a) the B domain is deleted (BDD Factor VIII);  
b) the synthetic nucleic acid sequence has a recognition site for an intracellular protease of the PACE/furin class, e.g., X-Arg-X-X-Arg (Molloy et al., *J. Biol. Chem.* 267:1639616401, 1992); a short-peptide linker, e.g., a two peptide linker, e.g., a leucine-glutamic acid peptide linker (LE), a three, or a four peptide linker, inserted at the heavy-light chain junction.

c) the synthetic nucleic acid sequence is introduced into a cell, e.g., a primary cell, a secondary cell a transformed or an immortalized cell line. Examples of an immortalized human cell line useful in the present method include, but are not limited to; a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL 2, CCL2.1, and CCL 2.2), a HL-60 cell (ATCC Accession No. CCL 240), a HT1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. CRL 1593), WI-38VA13 sub line 2R4 cells (ATCC Accession No. CLL 75.1), a CCRF-CEM cell (ATCC Accession No. CCL 119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., *Cancer Res.* 48: 5927-5932, 1988), as well as heterohybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line. In a preferred embodiment, the cell is a non-transformed cell. In various preferred embodiments, the cell is a mammalian cell, e.g., a primary or

secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells. In a most preferred embodiment, the cell is a secondary human fibroblast.

In a preferred embodiment, the synthetic nucleic acid sequence which encodes a factor VIII protein has at least one, preferably at least two, and most preferably, all of the characteristics a, b, and c described above.

In preferred embodiments, at least one non-common codon or less-common codon of the synthetic nucleic acid has been replaced by a common codon and the synthetic nucleic acid has one or more of the following properties: it has a continuous stretch of at least 90 codons all of which are common codons; it has a continuous stretch of common codons which comprise at least 33% of the codons of the synthetic nucleic acid sequence; at least 94% or more of the codons in the sequence encoding the protein are common codons and the synthetic nucleic acid sequence encodes a protein of at least about 90, 100, or 120 amino acids in length; it is at least 80 base pairs in length and which is free of unique restriction endonuclease sites that would occur in the message optimized sequence.

In a preferred embodiment, the number of non-common or less-common codons replaced is less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1.

In a preferred embodiment, the number of non-common or less-common codons remaining is less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1.

In preferred embodiments, the non-common and less-common codons replaced, taken together, are equal or less than 6%, 5%, 4%, 3%, 2%, 1% of the codons in the synthetic nucleic acid sequence.

In preferred embodiments, the non-common and less-common codons remaining, taken together, are equal or less than 6%, 5%, 4%, 3%, 2%, 1% of the codons in the synthetic nucleic acid sequence.

In a preferred embodiment, all non-common or less-common codons are replaced with common codons.

In a preferred embodiment, all non-common and less-common codons are replaced with common codons.

In various preferred embodiments, at least 94%, 95%, 96%, 97%, 98%, 99%, or all of the codons in the synthetic nucleic acid sequence are common codons.

Preferably, all of the codons in the synthetic nucleic acid sequence are common codons.

In preferred embodiments, the protein is expressed in a eukaryotic cell, e.g., a mammalian cell, e.g., a human cell, and the protein is a mammalian protein, e.g., a human protein.

In a preferred embodiment, the synthetic nucleic acid sequence includes a continuous stretch of common codons wherein the continuous stretch comprises at least 35%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 100% of codons in the synthetic nucleic acid sequence.

In another aspect, the invention features, a synthetic nucleic acid sequence which can direct the synthesis of an optimized message which encodes a Factor IX protein having one or more of the following characteristics:

- a) it has a PACE/furin, such as a X-Arg-X-X-Arg site, at a pro-peptide mature protein junction; or
- b) is inserted, e.g., via transfection, into a non-transformed cell, e.g., a primary or secondary cell, e.g., a primary human fibroblast.

In a preferred embodiment, the synthetic nucleic acid sequence which encodes a factor IX protein has at least one, and preferably, both of the characteristics a and b described above.

In preferred embodiments, at least one non-common codon or less-common codon of the synthetic nucleic acid has been replaced by a common codon and the synthetic nucleic acid has one or more of the following properties: it has a continuous stretch of at least 90 codons all of which are common codons; it has a continuous stretch of common codons which comprise at least 33% of the codons of the synthetic nucleic acid sequence; at least 94% or more of the codons in the sequence encoding the protein are common codons and the synthetic nucleic acid sequence encodes a protein of at least about 90, 100, or 120 amino acids in length; it is at least 80

base pairs in length and is free of unique restriction endonuclease sites that occur in the message optimized sequence.

In a preferred embodiment, the number of non-common or less-common codons replaced is less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1.

In a preferred embodiment, the number of non-common or less-common codons remaining is less than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1.

In preferred embodiments, the non-common and less-common codons replaced, taken together, are equal or less than 6%, 5%, 4%, 3%, 2%, 1% of the codons in the synthetic nucleic acid sequence.

In preferred embodiments, the non-common and less-common codons remaining, taken together, are equal or less than 6%, 5%, 4%, 3%, 2%, 1% of the codons in the synthetic nucleic acid sequence.

In a preferred embodiment, all non-common or less-common codons are replaced with common codons.

In a preferred embodiment, all non-common and less-common codons are replaced with common codons.

In various preferred embodiments, at least 94%, 95%, 96%, 97%, 98%, 99%, or all of the codons in the synthetic nucleic acid sequence are common codons.

Preferably, all of the codons in the synthetic nucleic acid sequence are common codons.

In preferred embodiments, the protein is expressed in a eukaryotic cell, e.g., a mammalian cell, e.g., a human cell, and the protein is a mammalian protein, e.g., a human protein.

In a preferred embodiment, the synthetic nucleic acid sequence includes a continuous stretch of common codons wherein the continuous stretch comprises at least 35%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 100% of codons in the synthetic nucleic acid sequence.

In another aspect, the invention features, a plasmid or a DNA construct, e.g., an expression plasmid or a DNA construct, which includes a synthetic nucleic acid sequence described herein.

In yet another aspect, the invention features, a synthetic nucleic acid sequence described herein introduced into the genome of an animal cell. In a preferred embodiment, the animal cell is a primate cell, e.g., a mammal cell, e.g., a human cell.

In still another aspect, the invention features, a cell harboring a synthetic nucleic acid sequence described herein, e.g., a cell from a primary or secondary cell strain, or a cell from a continuous cell line, e.g., a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL 2, CCL2.1, and CCL 2.2), a HL-60 cell (ATCC Accession No. CCL 240), a HT1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. CRL 1593), a WI-38VA13 sub line 2R4 cell (ATCC Accession No. CLL 75.1), a CCRF-CEM cell (ATCC Accession No. CCL 119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48: 5927-5932, 1988), as well as heterohybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be a cell line other than a human cell line, e.g., a CHO cell line. In a preferred embodiment, the cell is a non-transformed cell. In various preferred embodiments, the cell is a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells. In a most preferred embodiment, the cell is a secondary human fibroblast.

In another aspect, the invention features, a method for preparing a synthetic nucleic acid sequence encoding a protein which is, preferably, at least 90 codons in length, e.g., a synthetic nucleic acid sequence described herein. The method includes identifying non-common and less-common codons in the non-optimized gene encoding the protein and replacing at least, 94%, 95%, 96%, 97%, 98%, 99% or more of the non-common and less-common codons with a

common codon encoding the same amino acid as the replaced codon. Preferably, all non-common and less-common codons are replaced with common codons.

In a preferred embodiment, the synthetic nucleic acid sequence encodes a protein of at least about 90, 95, 100, 105, 110, 120, 130, 150, 200, 500, 700, 1000 or more codons in length.

In preferred embodiments, the protein is expressed in a eukaryotic cell, e.g., a mammalian cell, e.g., a human cell, and the protein is a mammalian protein, e.g., a human protein.

In another aspect, the invention features, a method for making a nucleic acid sequence which directs the synthesis of a optimized message of a protein of at least 90, 100, or 120 amino acids in length, e.g., a synthetic nucleic acid sequence described herein. The method includes: synthesizing at least two fragments of the nucleic acid sequence, wherein the two fragments encode adjoining portions of the protein and wherein both fragments are mRNA optimized, e.g., as described herein; and joining the two fragments such that a non-common codon is not created at a junction point, thereby making the mRNA optimized nucleic acid sequence.

In a preferred embodiment, the two fragments are joined together such that a unique restriction endonuclease site used to create the two fragments is not recreated at the junction point. In another preferred embodiment, the two fragments are joined together such that a unique restriction site is created.

In a preferred embodiment, the synthetic nucleic acid sequence encodes a protein of at least about 90, 95, 100, 105, 110, 120, 130, 150, 200, 500, 700, 1000 or more codons in length.

In a preferred embodiment, at least 3, 4, 5, 6, 7, 8, 9, 10 or more fragments of the nucleic acid sequence are synthesized.

In a preferred embodiment, the fragments are joined together by a fusion, e.g., a blunt end fusion.

In various preferred embodiments, at least 94%, 95%, 96%, 97%, 98%, 99%, or all of the codons in the synthetic nucleic acid sequence are common codons. Preferably, all of the codons in the synthetic nucleic acid sequence are common codons.

In preferred embodiments, the number of codons which are not common codons is equal to or less than 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1.



In preferred embodiments, each fragment is at least 30, 40, 50, 75, 100, 120, 150 or more codons in length.

In another aspect, the invention features, a method of providing a subject, e.g., a human, with a protein. The methods includes: providing a synthetic nucleic acid sequence that can direct the synthesis of an optimized message for a protein, e.g., a synthetic nucleic acid sequence described herein; introducing the synthetic nucleic acid sequence that directs the synthesis of an optimized message for a protein into the subject; and allowing the subject to express the protein, thereby providing the subject with the protein.

In preferred embodiments, the method further includes inserting the nucleic acid sequence that can direct the synthesis of an optimized message into a cell. The cell can be an autologous, allogeneic, or xenogeneic cell, but is preferably autologous. A preferred cell is a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells. The mRNA optimized synthetic nucleic acid sequence can be inserted into the cell *ex vivo* or *in vivo*. If inserted *ex vivo*, the cell can be introduced into the subject.

In preferred embodiments, at least 94%, 95%, 96%, , 97%, 98%, 99%, or all of the codons in the synthetic nucleic acid sequence are common codons. Preferably, all of the codons in the synthetic nucleic acid sequence are common codons.

In preferred embodiments, the number of codons which are not common codons is equal to or less than 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1.

The invention also features synthetic nucleic acid fragments which encode a portion of a protein. Such synthetic nucleic acid fragments are similar to the synthetic nucleic acid sequences of the invention except that they encode only a portion of a protein. Such nucleic acid fragments preferably encode at least 50, 60, 70, 80, 100, 110, 120, 130, 150, 200, 300, 400, 500, or more contiguous amino acids of the protein.

The invention also features transfected or infected primary and secondary somatic cells of vertebrate origin, particularly of mammalian origin, e.g., of human, mouse, or rabbit origins, e.g.,

primary human cells, secondary human cells, or primary or secondary rabbit cells. The cells are transfected or infected with exogenous synthetic nucleic acid, e.g., DNA, described herein. The synthetic nucleic acid can encode a protein, e.g., a therapeutic protein, e.g., an enzyme, a cytokine, a hormone, an antigen, an antibody, a clotting factor, e.g., Factor VIII, Factor IX, or a regulatory protein. The invention also includes methods by which primary and secondary cells are transfected or infected to include exogenous synthetic DNA, methods of producing clonal cell strains or heterogenous cell strains, and methods of gene therapy in which the transfected or infected primary or secondary cells are used. The synthetic nucleic acid directs the synthesis of an optimized message, e.g., an optimized message as described herein.

The present invention includes primary and secondary somatic cells, which have been transfected or infected with an exogenous synthetic nucleic acid described herein, which is stably integrated into their genomes or is expressed in the cells episomally. In preferred embodiments the cells are fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, cells comprising a formed element of the blood, muscle cells, other somatic cells which can be cultured, or somatic cell precursors. The resulting cells are referred to, respectively, as transfected or infected primary cells and transfected or infected secondary cells. The exogenous synthetic DNA encodes a protein, or a portion thereof, e.g., a therapeutic protein (e.g., Factor VIII or Factor IX). In an embodiment in which the exogenous synthetic DNA encodes a protein, or a portion thereof, to be expressed by the recipient cells, the resulting protein can be retained within the cell, incorporated into the cell membrane or secreted from the cell. In this embodiment, the exogenous synthetic DNA encoding the protein is introduced into cells along with additional DNA sequences sufficient for expression of the exogenous synthetic DNA in the cells. The additional DNA sequences may be of viral or non-viral origin. Primary cells modified to express exogenous synthetic DNA are referred to herein as transfected or infected primary cells, which include cells removed from tissue and placed on culture medium for the first time. Secondary cells modified to express or render available exogenous DNA are referred to herein as transfected or infected secondary cells.

Primary and secondary cells transfected or infected by the subject method, e.g., cloned cell strains, can be seen to fall into three types or categories: 1) cells which do not, as obtained, make or contain the therapeutic protein, 2) cells which make or contain the therapeutic protein but in lower quantities than normal (in quantities less than the physiologically normal lower

level) or in defective form, and 3) cells which make the therapeutic protein at physiologically normal levels, but are to be augmented or enhanced in their content or production. Examples of proteins that can be made by the present method include cytokines or clotting factors.

Exogenous synthetic DNA is introduced into primary or secondary cell by a variety of techniques. For example, a DNA construct which includes exogenous synthetic DNA encoding a therapeutic protein and additional DNA sequences necessary for expression in recipient cells can be introduced into primary or secondary cells by electroporation, microinjection, or other means (e.g., calcium phosphate precipitation, modified calcium phosphate precipitation, polybrene precipitation, liposome fusion, receptor-mediated DNA delivery). Alternatively, a vector, such as a retroviral or other vector which includes exogenous synthetic DNA can be used and cells can be genetically modified as a result of infection with the vector.

In addition to the exogenous synthetic DNA, transfected or infected primary and secondary cells may optionally contain DNA encoding a selectable marker, which is expressed and confers upon recipients a selectable phenotype, such as antibiotic resistance, resistance to a cytotoxic agent, nutritional prototrophy or expression of a surface protein. Its presence makes it possible to identify and select cells containing the exogenous DNA. A variety of selectable marker genes can be used, such as neo, gpt, dhfr, ada, pac, hyg, mdx and hisD.

Transfected or infected cells of the present invention are useful, as populations of transfected or infected primary cells or secondary cells, transfected or infected clonal cell strains, transfected or infected heterogenous cell strains, and as cell mixtures in which at least one representative cell of one of the three preceding categories of transfected or infected cells is present, (e.g., the mixture of cells contains essentially transfected or infected primary or secondary cells and may include untransfected or uninfected primary or secondary cells) as a delivery system for treating an individual with an abnormal or undesirable condition which responds to delivery of a therapeutic protein, which is either: 1) a therapeutic protein (e.g., a protein which is absent, underproduced relative to the individual's physiologic needs, defective, or inefficiently or inappropriately utilized in the individual, e.g., Factor VIII; or 2) a therapeutic protein with novel functions, such as enzymatic or transport functions. In the method of the present invention of providing a therapeutic protein, transfected or infected primary cells or secondary cells, clonal cell strains or heterogenous cell strains, are administered to an individual in whom the abnormal or undesirable condition is to be treated or prevented, in sufficient quanti-

ty and by an appropriate route, to express the exogenous synthetic DNA at physiologically relevant levels. A physiologically relevant level is one which either approximates the level at which the product is produced in the body or results in improvement of the abnormal or undesirable condition.

Clonal cell strains of transfected or infected secondary cells (referred to as transfected or infected clonal cell strains) expressing exogenous synthetic DNA (and, optionally, including a selectable marker gene) can be produced by the method of the present invention. The method includes the steps of: 1) providing a population of primary cells, obtained from the individual to whom the transfected or infected primary cells will be administered or from another source; 2) introducing into the primary cells or into secondary cells derived from primary cells a DNA construct which includes exogenous DNA as described above and the necessary additional DNA sequences described above, producing transfected or infected primary or secondary cells; 3) maintaining transfected or infected primary or secondary cells under conditions appropriate for their propagation; 4) identifying a transfected or infected primary or secondary cell; and 5) producing a colony from the transfected or infected primary or secondary cell identified in (4) by maintaining it under appropriate culture conditions until a desired number of cells is obtained. The desired number of clonal cells is a number sufficient to provide a therapeutically effective amount of product when administered to an individual, e.g., an individual with hemophilia A is provided with a population of cells that produce a therapeutically effective amount of Factor VIII, such that the condition is treated. The number of cells required for a given therapeutic dose depends on several factors including the expression level of the protein, the condition of the host animal and the limitations associated with the implantation procedure. In general, the number of cells required for implantation are in the range of  $1 \times 10^6$  to  $5 \times 10^9$ , and preferably  $1 \times 10^8$  to  $5 \times 10^8$ . In one embodiment of the method, the cell identified in (4) undergoes approximately 27 doublings (i.e., undergoes 27 cycles of cell growth and cell division) to produce 100 million clonal transfected or infected cells. In another embodiment of the method, exogenous synthetic DNA is introduced into genomic DNA by homologous recombination between DNA sequences present in the DNA construct and genomic DNA. In another embodiment, the exogenous synthetic DNA is present episomally in a transfected cell, e.g., primary or secondary cell.

In one embodiment of producing a clonal population of transfected secondary cells, a cell suspension containing primary or secondary cells is combined with exogenous synthetic DNA encoding a therapeutic protein and DNA encoding a selectable marker, such as the neo gene. The two DNA sequences are present on the same DNA construct or on two separate DNA constructs. The resulting combination is subjected to electroporation, generally at 250-300 volts with a capacitance of 960  $\mu$ Farads and an appropriate time constant (e.g., 14 to 20 m sec) for cells to take up the DNA construct. In an alternative embodiment, microinjection is used to introduce the DNA construct into primary or secondary cells. In either embodiment, introduction of the exogenous DNA results in production of transfected primary or secondary cells. The exogenous synthetic DNA introduced into the cell can be stably integrated into genomic DNA or is present episomally in the cell.

In the method of producing heterogenous cell strains of the present invention, the same steps are carried out as described for production of a clonal cell strain, except that a single transfected primary or secondary cell is not isolated and used as the founder cell. Instead, two or more transfected primary or secondary cells are cultured to produce a heterogenous cell strain. A heterogenous cell strain can also contain in addition to two or more transfected primary or secondary cells, untransfected primary or secondary cells.

The methods described herein have wide applicability in treating abnormal or undesired conditions and can be used to provide a variety of proteins in an effective amount to an individual. For example, they can be used to provide secreted proteins (with either predominantly systemic or predominantly local effects, e.g., Factor VIII and Factor IX), membrane proteins (e.g., for imparting new or enhanced cellular responsiveness, facilitating removal of a toxic product or for marking or targeting to a cell) or intracellular proteins (e.g., for affecting gene expression or producing autocrine effects).

A method described herein is particularly advantageous in treating abnormal or undesired conditions in that it: 1) is curative (one gene therapy treatment has the potential to last a patient's lifetime); 2) allows precise dosing (the patient's cells continuously determine and deliver the optimal dose of the required protein based on physiologic demands, and the stably transfected or infected cell strains can be characterized extensively in vitro prior to implantation, leading to accurate predictions of long term function in vivo); 3) is simple to apply in treating patients; 4)

eliminates issues concerning patient compliance (following a one-time gene therapy treatment, daily protein injections are no longer necessary); and 5) reduces treatment costs (since the therapeutic protein is synthesized by the patient's own cells, investment in costly protein production and purification is unnecessary).

As used herein, the term "optimized messenger RNA" refers to a synthetic nucleic acid sequence encoding a protein wherein at least one non-common codon or less-common codon in the sequence encoding the protein has been replaced with a common codon.

By "common codon" is meant the most common codon representing a particular amino acid in a human sequence. The codon frequency in highly expressed human genes is outlined below in Table 1. Common codons include: Ala (gcc); Arg (cgc); Asn (aac); Asp (gac); Cys (tgc); Gln (cag); Gly (ggc); His (cac); Ile (atc); Leu (ctg); Lys (aag); Pro (ccc); Phe (ttc); Ser (agc); Thr (acc); Tyr (tac); Glu (gag); and Val (gtg) (see Table 1). "Less-common codons" are codons that occurs frequently in humans but are not the common codon: Gly (ggg); Ile (att); Leu (etc); Ser (tcc); Val (gtc); and Arg (agg). All codons other than common codons and less-common codons are "non-common codons".

TABLE 1: Codon Frequency in Highly Expressed Human Genes

% occurrence			% occurrence		
Ala			Cys		
GC	C	53	TG	C	68
	T	17		T	32
	A	13	Gln		
	G	17	CA	A	12
Arg			G	88	
CG	C	37	Glu		
	T	7	GA	A	25
	A	6	G	75	
	G	21	Gly		
AG	A	10	GG	C	50
	G	18	T	12	
Asn			A	14	
AA	C	78	G	24	
	T	25	His		
Leu			CA	C	79
CT	C	26	T	21	
	T	5	Ile		
	A	3	AT	C	77
	G	58	T	18	
TT	A	2	A	5	
	G	6	Lys		
Lys			AA	A	18
AA	G	82	Ser		
			TC	C	28

				T	13
Pro				A	5
CC	C	48		G	9
	T	19	AG	C	34
	A	16		T	10
	G	17			
			Thr		
Phe			AC	C	57
TT	C	80		T	14
	T	20		A	14
				G	15
			Tyr		
			TA	C	74
				T	26
			Val		
			GT	C	25
				T	7
				A	5
				G	64

Codon frequency in Table 1 was calculated using the GCG program established by the University of Wisconsin Genetics Computer Group. Numbers represent the percentage of cases in which the particular codon is used.

The term "primary cell" includes cells present in a suspension of cells isolated from a vertebrate tissue source (prior to their being plated i.e., attached to a tissue culture substrate such as a dish or flask), cells present in an explant derived from tissue, both of the previous types of cells plated for the first time, and cell suspensions derived from these plated cells. The term secondary cell or cell strain refers to cells at all subsequent steps in culturing. That is, the first time a plated primary cell is removed from the culture substrate and replated (passaged), it is



referred to herein as a secondary cell, as are all cells in subsequent passages. Secondary cells are cell strains which consist of secondary cells which have been passaged one or more times. A cell strain consists of secondary cells that: 1) have been passaged one or more times; 2) exhibit a finite number of mean population doublings in culture; 3) exhibit the properties of contact-inhibited, anchorage dependent growth (anchorage-dependence does not apply to cells that are propagated in suspension culture); and 4) are not immortalized. A "clonal cell strain" is defined as a cell strain that is derived from a single founder cell. A "heterogenous cell strain" is defined as a cell strain that is derived from two or more founder cells.

The term "transfected cell" refers to a cell into which an exogenous synthetic nucleic acid sequence, e.g., a sequence which encodes a protein, is introduced. Once in the cell, the synthetic nucleic acid sequence can integrate into the recipients cells chromosomal DNA or can exist episomally. Standard transfection methods can be used to introduce the synthetic nucleic acid sequence into a cell, e.g., transfection mediated by liposome, polybrene, DEAE dextran-mediated transfection, electroporation, calcium phosphate precipitation or mircoinjection. The term "transfection" does not include delivery of DNA or RNA into a cell by a virus

The term "infected cell" refers to a cell into which an exogenous synthetic nucleic acid sequence, e.g., a sequence which encodes a protein, is introduced by a virus. Viruses known to be useful for gene transfer include an adenovirus, an adeno-associated virus, a herpes virus, a mumps virus, a poliovirus, a retrovirus, a Sindbis virus, a lentivirus and a vaccinia virus such as a canary pox virus. Other features and advantages of the invention will be apparent from the following detailed description and the claims.

#### Detailed Description

The drawings are first briefly described.

*Figure 1* is a schematic representation of domain structures of full-length and B-domain deleted human Factor VIII (hFVIII).

*Figure 2* is a schematic representation of full-length hFVIII.

*Figure 3* is a schematic representation of 5R BDD hFVIII expression plasmid pXF8.186.

*Figure 4* is a schematic representation of LE BDD hFVIII expression plasmid pXF8.61.

*Figure 5* is a schematic representation of the fourteen fragments (Fragments A-Fragment N) assembled to construct pXF8.61.

*Figure 6* is a schematic representation of the assembly of pXF8.61.

*Figure 7* depicts the nucleotide sequence and the corresponding amino acid sequence of the LE B-domain-deleted-Factor VIII (FVIII)insert contained in pAM1-1 (SEQ ID NO:1).

*Figure 8* is a schematic representation of the fragments assembled to construct pXF8.186.

*Figure 9* depicts the nucleotide sequence and the corresponding amino acid sequence of the 5Arg B-domain-deleted-FVIII insert (SEQ ID NO:2).

*Figure 10* is a schematic representation of the Factor VIII expression plasmid, pXF8.36. The cytomegalovirus immediate early I (CMV) promoter is depicted as a lightly shaded box. Positions of splice donor (SD) and splice acceptor (SA) sites are indicated below the shaded box. The Factor VIII cDNA sequence is depicted as a solid dark box. The hGH 3'UTS region is depicted as an open box. The new expression cassette is depicted as a shaded box with an arrowhead which corresponds to the direction of transcription. The thin dark line represents the plasmid backbone sequences. The position and direction of transcription of the  $\beta$ -lactamase gene (*amp*) is indicated by the solid boxed arrow.

*Figure 11* is a schematic representation of the Factor VIII expression plasmid, pXF8.38. The cytomegalovirus immediate early I (CMV) promoter is depicted as a lightly shaded box. Positions of splice donor (SD) and splice acceptor (SA) sites are indicated below the shaded box. The Factor VIII cDNA sequence is depicted as a solid dark box. The hGH 3'UTS region is depicted as an open box. The *neo* expression cassette is depicted as a shaded box with an arrowhead which corresponds to the direction of transcription. The thin dark line represents the plasmid backbone sequences. The position and direction of transcription of the  $\beta$ -lactamase gene (*amp*) is indicated by the solid boxed arrow.

*Figure 12* is a schematic representation of the Factor VIII expression plasmid, pXF8.269. The collagen (I)  $\alpha$  2 promoter is depicted as a striped box. The region representing aldolase-derived 5' untranslated sequences are depicted as a lightly shaded box. Positions of splice donor (SD) and splice acceptor (SA) sites are indicated below the shaded box. The Factor VIII cDNA sequence is depicted as a solid dark box. The hGH 3'UTS region is depicted as an open box. The *neo* expression cassette is depicted as a shaded box with an arrowhead which corresponds to the direction of transcription. The thin dark line represents the plasmid backbone

sequences. The position and direction of transcription of the  $\beta$ -lactamase gene (*amp*) is indicated by the solid boxed arrow.

*Figure 13* is a schematic representation of the Factor VIII expression plasmid, pXF8.224. The collagen (I)  $\alpha$  2 promoter is depicted as a striped box. The region representing aldolase-derived 5' untranslated sequences are depicted as a lightly shaded box. Positions of splice donor (SD) and splice acceptor (SA) sites are indicated below the shaded box. The Factor VIII cDNA sequence is depicted as a solid dark box. The hGH 3'UTS region is depicted as an open box. The neo expression cassette is depicted as a shaded box with an arrowhead which corresponds to the direction of transcription. The thin dark line represents the plasmid backbone sequences. The position and direction of transcription of the  $\beta$ -lactamase gene (*amp*) is indicated by the solid boxed arrow.

### Message Optimization

Methods of the invention are directed to optimized messages and synthetic nucleic acid sequences which direct the production of optimized mRNAs. An optimized mRNA can direct the synthesis of a protein of interest, e.g., a human protein, e.g. a human Factor VIII. A message for a protein of interest, e.g., human Factor VIII, can be optimized as described herein, e.g., by replacing at least 94%, 95%, 96%, 97%, 98%, 99%, and preferably all of the non-common codons or less-common codons with a common codon encoding the same amino acid as outlined in Table 1.

The coding region of a synthetic nucleic acid sequence can include the sequence "cg" without any discrimination, if the sequence is found in the common codon for that amino acid. Alternatively, the sequence "cg" can be limited in various regions, e.g., the first 20% of the coding sequence can be designed to have a low incidence of the sequence "cg".

Optimizing a message (and its synthetic DNA sequence) can negatively or positively affect gene expression or protein production. For example, replacing a less-common codon with a more common codon may affect the half life of the mRNA or alter its structure by introducing a secondary structure that interferes with translation of the message. It may therefore be necessary, in certain instances, to alter the optimized message.

All or a portion of a message (or its gene) can be optimized. In some cases the desired modulation of expression is achieved by optimizing essentially the entire message. In other cases, the desired modulation will be achieved by optimizing part but not all of the message or gene.

The codon usage of any coding sequence can be adjusted to achieve a desired property, for example high levels of expression in a specific cell type. The starting point for such an optimization may be a coding sequence with 100% common codons, or a coding sequence which contains a mixture of common and non-common codons.

Two or more candidate sequences that differ in their codon usage are generated and tested to determine if they possess the desired property. Candidate sequences may be evaluated initially by using a computer to search for the presence of regulatory elements, such as silencers or enhancers, and to search for the presence of regions of coding sequence which could be converted into such regulatory elements by an alteration in codon usage. Additional criteria may include enrichment for particular nucleotides, e.g., A, C, G or U, codon bias for a particular amino acid, or the presence or absence of particular mRNA secondary or tertiary structure. Adjustment to the candidate sequence can be made based on a number of such criteria.

Promising candidate sequences are constructed and then evaluated experimentally. Multiple candidates may be evaluated independently of each other, or the process can be iterative, either by using the most promising candidate as a new starting point, or by combining regions of two or more candidates to produce a novel hybrid. Further rounds of modification and evaluation can be included.

Modifying the codon usage of a candidate sequence can result in the creation or destruction of either a positive or negative element. In general, a positive element refers to any element whose alteration or removal from the candidate sequence could result in a decrease in expression of the therapeutic protein, or whose creation could result in an increase in expression of a therapeutic protein. For example, a positive element can include an enhancer, a promoter, a downstream promoter element, a DNA binding site for a positive regulator (e.g., a transcriptional activator), or a sequence responsible for imparting or removing mRNA secondary or tertiary structure. A negative element refers to any element whose alteration or removal from the candidate sequence could result in an increase in expression of the therapeutic protein, or whose creation would result in a decrease in expression of the therapeutic protein. A negative element

includes a silencer, a DNA binding site for a negative regulator (e.g., a transcriptional repressor), a transcriptional pause site, or a sequence that is responsible for imparting or removing mRNA secondary or tertiary structure. In general, a negative element arises more frequently than a positive element. Thus, any change in codon usage that results in an increase in protein expression is more likely to have arisen from the destruction of a negative element rather than the creation of a positive element. In addition, alteration of the candidate sequence is more likely to destroy a positive element than create a positive element. In one embodiment, a candidate sequence is chosen and modified so as to increase the production of a therapeutic protein. The candidate sequence can be modified, e.g., by sequentially altering the codons or by randomly altering the codons in the candidate sequence. A modified candidate sequence is then evaluated by determining the level of expression of the resulting therapeutic protein or by evaluating another parameter, e.g., a parameter correlated to the level of expression. A candidate sequence which produces an increased level of a therapeutic protein as compared to an unaltered candidate sequence is chosen.

In another approach, one or a group of codons can be modified, e.g., without reference to protein or message structure and tested. Alternatively, one or more codons can be chosen on a message-level property, e.g., location in a region of predetermined, e.g., high or low, GC or AU content, location in a region having a structure such as an enhancer or silencer, location in a region that can be modified to introduce a structure such as an enhancer or silencer, location in a region having, or predicted to have, secondary or tertiary structure, e.g., intra-chain pairing, inter-chain pairing, location in a region lacking, or predicted to lack, secondary or tertiary structure, e.g., intra-chain or inter-chain pairing. A particular modified region is chosen if it produces the desired result.

Methods which systematically generate candidate sequences are useful. For example, one or a group, e.g., a contiguous block of codons, at various positions of a synthetic nucleic acid sequence can be replaced with common codons (or with non common codons, if for example, the starting sequence has been optimized) and the resulting sequence evaluated. Candidates can be generated by optimizing (or de-optimizing) a given "window" of codons in the sequence to generate a first candidate, and then moving the window to a new position in the sequence, and optimizing (or de-optimizing) the codons in the new position under the window to provide a second candidate. Candidates can be evaluated by determining the level of expression they

provide, or by evaluating another parameter, e.g., a parameter correlated to the level of expression. Some parameters can be evaluated by inspection or computationally, e.g., the possession or lack thereof of high or low GC or AU content; a sequence element such as an enhancer or silencer; secondary or tertiary structure, e.g., intra-chain or inter-chain pairing

Thus, hybrid messages, i.e., messages having a region which is optimized and a region which is not optimized, can be evaluated to determine if they have a desired property. The evaluation can be effected by, e.g., synthesizing the candidate message or messages, and determining a property such as its level of expression. Such a determination can be made in a cell-free system or in a cell-based system. The generation and testing of one or more candidates can also be performed, by computational methods, e.g., on a computer. For example, a computer program can be used to generate a number of candidate messages and those messages analysed by a computer program which predicts the existence of primary structure elements or secondary or tertiary structure.

A candidate message can be generated by dividing a region into subregions and optimizing each subregion. An optimized subregion is then combined with a non-optimized subregion to produce a candidate. For example, a region is divided into three subregions, a, b and c, each of which is then optimized to provide optimized subregions a', b' and c'. The optimized subregions, a', b', and c' can then be combined with one or more of the non-optimized subregions, e.g., a, b and c. For example, ab'c could be formed and tested. Different combinations of optimized and non-optimized subregions can be generated. By evaluating a series of such hybrid candidate sequences, it is possible to analyze the effect of modification of different subregions and, e.g., to define the particular version of each subregion that contributes most to the desired property. A preferred candidate can include the versions of each subregion that performed best in a series of such experiments.

An algorithm for creating an optimized candidate sequence is as follows:

1. Provide a message sequence (an entire message or a portion thereof). Go to step 2.
2. Generate a novel candidate sequence by modifying the codon usage of a candidate sequence by using, the most promising candidate sequence previously identified, or

by combining regions of two or more candidates previously identified to produce a novel hybrid. Go to step 3.

3. Evaluate the candidate sequence and determine if it has a predetermined property. If the candidate has the predetermined property, then proceed to step 4, otherwise proceed to step 2.
4. Use the candidate sequence as an optimized message.

Methods can include first optimizing a mammalian synthetic nucleic acid sequence which encodes a protein of interest or a portion thereof, e.g., human Factor VIII, etc. The synthetic nucleic acid sequence can be optimized such that 94%, 95%, 96%, 97%, 98%, 99%, or all, of the codons of the synthetic DNA are replaced with common codons. The next step involves determining the amount of protein produced as a result of message optimization compared to the amount of protein produced using the wild type sequence. In instances where the amount of protein produced is not of the desired or expected level, it may be desirable to replace one or more of the common codons of the protein coding region with a less-common codon or non-common codon. A mammalian optimized message which is re-engineered such that common codons are replaced with less-common or non-common mammalian codons, or common codons of other eukaryotic species can result in at least 1%, 5%, 10%, 20% or more of the common codons being replaced. Re-engineering the optimized message can be done, for example, systematically by replacing a single common codon with a less-common or non-common codon. Alternatively, a block of 2, 4, 6, 10, 20, 40 or more codons may be replaced with a less-common or non-common codons. The level of protein produced by these "re-engineered optimized" messages determines which re-engineered optimized message is chosen.

Another approach of optimizing a message for increased protein expression includes altering the specific nucleotide content of an optimized synthetic nucleic acid sequence. The synthetic nucleic acid sequence can be altered by increasing or decreasing specific nucleotide(s) content, e.g., G, C, A, T, GC or AT content of the sequence. Increasing or decreasing the specific nucleotide content of a synthetic nucleotide sequence can be done by substituting the nucleotide of interest with another nucleotide. For example, a sequence that has a large number of codons that have a high GC content, e.g., glycine (GGC), can be substituted with codons that





antithrombin III; DNase;  $\alpha$ -galactosidase; tyrosine hydroxylase; apolipoprotein E; apolipoprotein A-I; globins; low density lipoprotein receptor; IL-2 receptor; IL-2 antagonists; alpha-1 antitrypsin; immune response modifiers; soluble CD4; a protein expressed under disease conditions; and proteins encoded by viruses, e.g., proteins which are encoded by a virus (including a retrovirus) which are expressed in mammalian cells post-infection.

In preferred embodiments, the synthetic nucleic acid sequence can express its protein, e.g., a eukaryotic e.g., mammalian, protein, at a level which is at least 110%, 150%, 200%, 500%, 1,000%, 5,000% or even 10,000% of that expressed by nucleic acid sequence that has not been optimized. This comparison can be made, e.g., in an *in vitro* mammalian cell culture system wherein the non-optimized and optimized sequence are expressed under the same conditions (e.g., the same cell type, same culture conditions, same expression vector).

Suitable cell culture systems for measuring expression of the synthetic nucleic acid sequence and corresponding non-optimized nucleic acid sequence are known in the art. (e.g., the pBS phagemic vectors, Stratagene, La Jolla, CA) and are described in, for example, the standard molecular biology reference books. Vectors suitable for expressing the synthetic and non-optimized nucleic acid sequences encoding the protein of interest are described below and in the standard reference books described below. Expression can be measured using an antibody specific for the protein of interest (e.g., ELISA). Such antibodies and measurement techniques are known to those skilled in the art.

In a preferred embodiment the protein is a human protein. In more preferred embodiments, the protein is human Factor VIII and the protein is a B domain deleted human Factor VIII. In another preferred embodiment the protein is B domain deleted human Factor VIII with a sequence which includes a recognition site for an intracellular protease of the PACE/furin class, such as X-ARG-X-X-ARG site, a short-peptide linker, e.g., a two peptide linker, e.g., a leucine-glutamic acid peptide linker (LE), or a three, or four peptide linker, inserted at the heavy-light chain junction (see Fig. 1).

A large fraction of the codons in the human messages encoding Factor VIII and Factor IX are non-common codons or less common codons. Replacement of at least 98% of these codons with common codons will yield nucleic acid sequences capable of higher level expression in a cell culture. Preferably, all of the codons are replaced with common codons and such replacement results in at least a 5 fold, more preferably a 10 fold and most preferably a 20

fold increase in expression when compared to an expression of the corresponding native sequence in the same expression system.

The synthetic nucleic acid sequences of the invention can be introduced into the cells of a living organism. The sequences can be introduced directly, e.g., via homologous recombination, or via a vector. For example, DNA constructs or vectors can be used to introduce a synthetic nucleic acid sequence into cells of a living organism for gene therapy. See, e.g., U.S. Patent No. 5,460,959; and co-pending U.S. applications USSN 08/334,797; USSN 08/231,439; USSN 08/334,455; and USSN 08/928,881 which are hereby expressly incorporated by reference in their entirety.

#### Transfected or Infected Cells

Primary and secondary cells to be transfected can be obtained from a variety of tissues and include cell types which can be maintained and propagated in culture. For example, primary and secondary cells which can be transfected include fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), endothelial cells, glial cells, neural cells, a cell comprising a formed element of the blood (e.g., lymphocytes, bone marrow cells), muscle cells and precursors of these somatic cell types. Primary cells are preferably obtained from the individual to whom the transfected primary or secondary cells are administered. However, primary cells may be obtained from a donor (other than the recipient) of the same species or another species (e.g., mouse, rat, rabbit, cat, dog, pig, cow, bird, sheep, goat, horse).

Primary or secondary cells of vertebrate, particularly mammalian, origin can be transfected with exogenous synthetic DNA encoding a therapeutic protein and produce an encoded therapeutic protein stably and reproducibly, both *in vitro* and *in vivo*, over extended periods of time. In addition, the transfected primary and secondary cells can express the encoded product in vivo at physiologically relevant levels, cells can be recovered after implantation and, upon reculturing, to grow and display their preimplantation properties.

The transfected primary or secondary cells may also include DNA encoding a selectable marker which confers a selectable phenotype upon them, facilitating their identification and isolation. Methods for producing transfected primary, secondary cells which stably express exogenous synthetic DNA, clonal cell strains and heterogenous cell strains of such transfected cells, methods of producing the clonal and heterogenous cell strains, and methods of treating or

preventing an abnormal or undesirable condition through the use of populations of transfected primary or secondary cells are part of the present invention. Primary and secondary cells which can be transfected include fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), endothelial cells, glial cells, neural cells, a cell comprising a formed element of the blood (e.g., a lymphocyte, a bone marrow cell), muscle cells and precursors of these somatic cell types. Primary cells are preferably obtained from the individual to whom the transfected primary or secondary cells are administered. However, primary cells may be obtained from a donor (other than the recipient) of the same species or another species (e.g., mouse, rat, rabbit, cat, dog, pig, cow, bird, sheep, goat, horse). Transformed or immortalized cells can also be used e.g., a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL 2, CCL2.1, and CCL 2.2), a HL-60 cell (ATCC Accession No. CCL 240), a HT1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. CRL 1593), WI-38VA13 sub line 2R4 cells (ATCC Accession No. CLL 75.1), a CCRF-CEM cell (ATCC Accession No. CCL 119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48: 5927-5932, 1988), as well as heterohybridoma cells produced by fusion of human cells and cells of another species.. In another embodiment, the immortalized cell line can be a cell line other than a human cell line, e.g., a CHO cell line. In a preferred embodiment, the cell is a non-transformed cell. In various preferred embodiments, the cell is a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells. In a most preferred embodiment, the cell is a secondary human fibroblast.

Alternatively, DNA can be delivered into any of the cell types discussed above by a viral vector infection. Viruses known to be useful for gene transfer include adenoviruses, adeno-associated virus, herpes virus, mumps virus, poliovirus, retroviruses, Sindbis virus, and vaccinia

virus such as canary pox virus. Use of viral vectors is well known in the art: see e.g., Robbins and Ghizzani, "Viral Vectors for Gene Therapy", *Mol. Med. Today* 1:410-417, 1995. A cell which has an exogenous DNA introduced into it by a viral vector is referred to as an "infected cell"

The invention also includes the genetic manipulation of a cell which normally produces a therapeutic protein. In this instance, the cell is manipulated such that the endogenous sequence which encodes the therapeutic protein is replaced with an optimized coding sequence, e.g., by homologous recombination.

### Exogenous Synthetic DNA

Exogenous synthetic DNA incorporated into primary or secondary cells by the present method can be a synthetic DNA which encodes a protein, or a portion thereof, useful to treat an existing condition or prevent it from occurring.

Synthetic DNA incorporated into primary or secondary cells can be an entire gene encoding an entire desired protein or a gene portion which encodes, for example, the active or functional portion(s) of the protein. The protein can be, for example, a hormone, a cytokine, an antigen, an antibody, an enzyme, a clotting factor, e.g., Factor VIII or Factor XI, a transport protein, a receptor, a regulatory protein, a structural protein, or a protein which does not occur in nature. The DNA can be produced, using genetic engineering techniques or synthetic processes.

The DNA introduced into primary or secondary cells can encode one or more therapeutic proteins. After introduction into primary or secondary cells, the exogenous synthetic DNA is stably incorporated into the recipient cell's genome (along with the additional sequences present in the DNA construct used), from which it is expressed or otherwise functions. Alternatively, the exogenous synthetic DNA may exist episomally within the primary or secondary cells.

### Selectable Markers

A variety of selectable markers can be incorporated into primary or secondary cells. For example, a selectable marker which confers a selectable phenotype such as drug resistance, nutritional auxotrophy, resistance to a cytotoxic agent or expression of a surface protein, can be used. Selectable marker genes which can be used include neo, gpt, dhfr, ada, pac (puromycin),

hyg and hisD. The selectable phenotype conferred makes it possible to identify and isolate recipient primary or secondary cells.

### DNA Constructs

DNA constructs, which include exogenous synthetic DNA and, optionally, DNA encoding a selectable marker, along with additional sequences necessary for expression of the exogenous synthetic DNA in recipient primary or secondary cells, are used to transfect primary or secondary cells in which the encoded protein is to be produced. Alternatively, infectious vectors, such as retroviral, herpes, lentivirus, adenovirus, adenovirus-associated, mumps and poliovirus vectors, can be used for this purpose.

A DNA construct which includes the exogenous synthetic DNA and additional sequences, such as sequences necessary for expression of the exogenous synthetic DNA, can be used. A DNA construct which includes DNA encoding a selectable marker, along with additional sequences, such as a promoter, polyadenylation site and splice junctions, can be used to confer a selectable phenotype upon introduction into primary or secondary cells. The two DNA constructs are introduced into primary or secondary cells, using methods described herein. Alternatively, one DNA construct which includes exogenous synthetic DNA, a selectable marker gene and additional sequences (e.g., those necessary for expression of the exogenous synthetic DNA and for expression of the selectable marker gene) can be used.

### Transfection of Primary or Secondary Cells and Production of Clonal or Heterogenous Cell Strains

Vertebrate tissue can be obtained by standard methods such as punch biopsy or other surgical methods of obtaining a tissue source of the primary cell type of interest. For example, punch biopsy is used to obtain skin as a source of fibroblasts or keratinocytes. A mixture of primary cells is obtained from the tissue, using known methods, such as enzymatic digestion. If enzymatic digestion is used, enzymes such as collagenase, hyaluronidase, dispase, pronase, trypsin, elastase and chymotrypsin can be used.

The resulting primary cell mixture can be transfected directly or it can be cultured first, removed from the culture plate and resuspended before transfection is carried out. Primary cells or secondary cells are combined with exogenous synthetic DNA to be stably integrated into their

genomes and, optionally, DNA encoding a selectable marker, and treated in order to accomplish transfection. The exogenous synthetic DNA and selectable marker-encoding DNA are each on a separate construct or on a single construct and an appropriate quantity of DNA to ensure that at least one stably transfected cell containing and appropriately expressing exogenous DNA is produced. In general, 0.1 to 500 ug DNA is used.

Primary or secondary cells, can be transfected by electroporation. Electroporation is carried out at appropriate voltage and capacitance (and time constant) to result in entry of the DNA construct(s) into the primary or secondary cells. Electroporation can be carried out over a wide range of voltages (e.g., 50 to 2000 volts) and capacitance values (e.g., 60-300  $\mu$ Farads). Total DNA of approximately 0.1 to 500 ug is generally used.

Primary or secondary cells can be transfected using microinjection. Alternatively, known methods such as calcium phosphate precipitation, modified calcium phosphate precipitation and polybrene precipitation, liposome fusion and receptor-mediated gene delivery can be used to transfect cells. A stably, transfected cell is isolated and cultured and subcultivated, under culturing conditions and for sufficient time, to propagate the stably transfected secondary cells and produce a clonal cell strain of transfected secondary cells. Alternatively, more than one transfected cell is cultured and subcultured, resulting in production of a heterogenous cell strain.

Transfected primary or secondary cells undergo a sufficient number of doublings to produce either a clonal cell strain or a heterogenous cell strain of sufficient size to provide the therapeutic protein to an individual in effective amounts. In general, for example, 0.1 cm<sup>2</sup> of skin is biopsied and assumed to contain 100,000 cells; one cell is used to produce a clonal cell strain and undergoes approximately 27 doublings to produce 100 million transfected secondary cells. If a heterogenous cell strain is to be produced from an original transfected population of approximately 100,000 cells, only 10 doublings are needed to produce 100 million transfected cells.

The number of required cells in a transfected clonal or heterogenous cell strain is variable and depends on a variety of factors, including but not limited to, the use of the transfected cells, the functional level of the exogenous DNA in the transfected cells, the site of implantation of the transfected cells (for example, the number of cells that can be used is limited by the anatomical

site of implantation), and the age, surface area, and clinical condition of the patient. To put these factors in perspective, to deliver therapeutic levels of human growth hormone in an otherwise healthy 10 kg patient with isolated growth hormone deficiency, approximately one to five hundred million transfected fibroblasts would be necessary (the volume of these cells is about that of the very tip of the patient's thumb).

#### Episomal Expression of Exogenous Synthetic DNA

DNA sequences that are present within the cell yet do not integrate into the genome are referred to as episomes. Recombinant episomes may be useful in at least three settings: 1) if a given cell type is incapable of stably integrating the exogenous synthetic DNA; 2) if a given cell type is adversely affected by the integration of synthetic DNA; and 3) if a given cell type is capable of improved therapeutic function with an episomal rather than integrated synthetic DNA.

Using transfection and culturing as described herein, exogenous synthetic DNA in the form of episomes can be introduced into vertebrate primary and secondary cells. Plasmids can be converted into such an episome by the addition DNA sequences for the Epstein-Barr virus origin of replication and nuclear antigen (Yates, J.L. Nature 319:780-7883 (1985)). Alternatively, vertebrate autonomously replicating sequences can be introduced into the construct (Weidle, U.H. Gene 73(2):427-437 (1988). These and other episomally derived sequences can also be included in DNA constructs without selectable markers, such as pXGH5 (Selden et al., Mol Cell Biol. 6:3173-3179, 1986). The episomal synthetic exogenous DNA is then introduced into primary or secondary vertebrate cells as described in this application (if a selective marker is included in the episome a selective agent is used to treat the transfected cells).

#### Implantation of Clonal Cell Strain or Heterogenous Cell Strain of Transfected Secondary Cells

The transfected cells produced as described above can be introduced into an individual to whom the therapeutic protein is to be delivered, using known methods. The clonal cell strain or heterogenous cell strain is then introduced into an individual, using known methods, using various routes of administration and at various sites (e.g., renal subcapsular, subcutaneous, central nervous system (including intrathecal), intravascular, intrahepatic, intrasplanchnic, intraperitoneal (including intraomental, or intramuscular implantation). In a preferred embodiment, the clonal cell strain or heterogeneous cell strain is introduced into the omentum.

The omentum is a membranous structure containing a sheet of fat. Usually, the omentum is a fold of peritoneum extending from the stomach to adjacent abdominal organs. The greater omentum is attached to the inferior edge of the stomach and hangs down in front of the intestines.

The other edge is attached to the transverse colon. The lesser omentum is attached to the superior edge of the stomach and extends to the undersurface of the liver. The cells may be introduced into any part of the omentum by surgical implantation, laparoscopy or direct injection, e.g., via CT-guided needle or ultrasound. Once implanted in the individual, the cells produce the therapeutic product encoded by the exogenous synthetic DNA or are affected by the exogenous synthetic DNA itself. For example, an individual who has been diagnosed with Hemophilia A, a bleeding disorder that is caused by a deficiency in Factor VIII, a protein normally found in the blood, is a candidate for a gene therapy treatment. In another example, an individual who has been diagnosed with Hemophilia B, a bleeding disorder that is caused by a deficiency in Factor IX, a protein normally found in the blood, is a candidate for a gene therapy treatment. The patient has a small skin biopsy performed; this is a simple procedure which can be performed on an out-patient basis. The piece of skin, approximately the size of a matchhead, is taken, for example, from under the arm and requires about one minute to remove. The sample is processed, resulting in isolation of the patient's cells and genetically engineered to produce the missing Factor IX or Factor VIII. Based on the age, weight, and clinical condition of the patient, the required number of cells are grown in large-scale culture. The entire process requires 4-6 weeks and, at the end of that time, the appropriate number, e.g., approximately 100-500 million genetically-engineered cells are introduced into the individual, once again as an outpatient (e.g., by injecting them back under the patient's skin). The patient is now capable of producing his or her own Factor IX or Factor VIII and is no longer a hemophiliac.

A similar approach can be used to treat other conditions or diseases. For example, short stature can be treated by administering human growth hormone to an individual by implanting primary or secondary cells which express human growth hormone; anemia can be treated by administering erythropoietin (EPO) to an individual by implanting primary or secondary cells which express EPO; or diabetes can be treated by administering glucagon-like peptide-1 (GLP-1) to an individual by implanting primary or secondary cells which express GLP-1. A lysosomal storage disease (LSD) can be treated by this approach. LSD's represent a group of at least 41 distinct genetic diseases, each one representing a deficiency of a particular protein that is



involved in lysosomal biogenesis. A particular LSD can be treated by administering a lysosomal enzyme to an individual by implanting primary or secondary cells which express the lysosomal enzyme, e.g., Fabry Disease can be treated by administering  $\alpha$ -galactosidase to an individual by implanting primary or secondary cells which express  $\alpha$ -galactosidase; Gaucher disease can be treated by administering  $\beta$ -glucocereamidase to an individual by implanting primary or secondary cells which express  $\beta$ -glucocereamidase; MPS (mucopolysaccharidosis) type 1 (Hurley-Scheie syndrome) can be treated by administering  $\alpha$ -iduronidase to an individual by implanting primary or secondary cells which express  $\alpha$ -iduronidase; MPS type II (Hunter syndrome) can be treated by administering  $\alpha$ -L-iduronidase to an individual by implanting primary or secondary cells which express  $\alpha$ -L-iduronidase; MPS type III-A (Sanfilippo A syndrome) can be treated by administering glucosamine-N-sulfatase to an individual by implanting primary or secondary cells which express glucosamine-N-sulfatase; MPS type III-B (Sanfilippo B syndrome) can be treated by administering alpha-N-acetylglucosaminidase to an individual by implanting primary or secondary cells which express alpha-N-acetylglucosaminidase; MPS type III-C (Sanfilippo C syndrome) can be treated by administering acetylcoenzyme A: $\alpha$ -glucosmainide-N-acetyltransferase to an individual by implanting primary or secondary cells which express acetylcoenzyme A: $\alpha$ -glucosmainide-N-acetyltransferase; MPS type III-D (Sanfilippo D syndrome) can be treated by administering N-acetylglucosamine-6-sulfatase to an individual by implanting primary or secondary cells which express N-acetylglucosamine-6-sulfatase; MPS type IV-A (Morquio A syndrome) can be treated by administering N-Acetylglucosamine-6-sulfatase to an individual by implanting primary or secondary cells which express N-acetylglucosamine-6-sulfatase; MPS type IV-B (Morquio B syndrome) can be treated by administering  $\beta$ -galactosidase to an individual by implanting primary or secondary cells which express  $\beta$ -galactosidase; MPS type VI (Maroteaux-Lary syndrome) can be treated by administering N-acetylglactosamine-6-sulfatase to an individual by implanting primary or secondary cells which express N-acetylglactosamine-6-sulfatase; MPS type VII (Sly syndrome) can be treated by administering  $\beta$ -glucuronidase to an individual by implanting primary or secondary cells which express  $\beta$ -glucuronidase.

The cells used for implantation will generally be patient-specific genetically-engineered cells. It is possible, however, to obtain cells from another individual of the same species or from

a different species. Use of such cells might require administration of an immunosuppressant, alteration of histocompatibility antigens, or use of a barrier device to prevent rejection of the implanted cells. For many diseases, this will be a one-time treatment and, for others, multiple gene therapy treatments will be required.

#### Uses of Transfected or Infected Primary and Secondary Cells and Cell Strains

Transfected or infected primary or secondary cells or cell strains have wide applicability as a vehicle or delivery system for therapeutic proteins, such as enzymes, hormones, cytokines, antigens, antibodies, clotting factors, anti-sense RNA, regulatory proteins, transcription proteins, receptors, structural proteins, novel (non-optimized) proteins and nucleic acid products, and engineered DNA. For example, transfected primary or secondary cells can be used to supply a therapeutic protein, including, but not limited to, Factor VIII, Factor IX, erythropoietin, alpha-1 antitrypsin, calcitonin, glucocerebrosidase, growth hormone, low density lipoprotein (LDL), receptor IL-2 receptor and its antagonists, insulin, globin, immunoglobulins, catalytic antibodies, the interleukins, insulin-like growth factors, superoxide dismutase, immune responder modifiers, parathyroid hormone and interferon, nerve growth factors, tissue plasminogen activators, and colony stimulating factors. Alternatively, transfected primary and secondary cells can be used to immunize an individual (i.e., as a vaccine).

The wide variety of uses of cell strains of the present invention can perhaps most conveniently be summarized as shown below. The cell strains can be used to deliver the following therapeutic products.

1. a secreted protein with predominantly systemic effects;
2. a secreted protein with predominantly local effects;
3. a membrane protein imparting new or enhanced cellular responsiveness;
4. membrane protein facilitating removal of a toxic product;
5. a membrane protein marking or targeting a cell;
6. an intracellular protein;
7. an intracellular protein directly affecting gene expression; and
8. an intracellular protein with autocrine effects.

Transfected or infected primary or secondary cells can be used to administer therapeutic proteins (e.g., hormones, enzymes, clotting factors) which are presently administered intravenously, intramuscularly or subcutaneously, which requires patient cooperation and, often, medical staff participation. When transfected or infected primary or secondary cells are used, there is no need for extensive purification of the polypeptide before it is administered to an individual, as is generally necessary with an isolated polypeptide. In addition, transfected or infected primary or secondary cells of the present invention produce the therapeutic protein as it would normally be produced.

An advantage to the use of transfected or infected primary or secondary cells is that by controlling the number of cells introduced into an individual, one can control the amount of the protein delivered to the body. In addition, in some cases, it is possible to remove the transfected or infected cells if there is no longer a need for the product. A further advantage of treatment by use of transfected or infected primary or secondary cells of the present invention is that production of the therapeutic product can be regulated, such as through the administration of zinc, steroids or an agent which affects transcription of a protein, product or nucleic acid product or affects the stability of a nucleic acid product.

#### Transgenic animals

A number of methods have been used to obtain transgenic, non-human mammals. A transgenic non-human mammal refers to a mammal that has gained an additional gene through the introduction of an exogenous synthetic nucleic acid sequence, i.e., transgene, into its own cells (e.g., both the somatic and germ cells), or into an ancestor's germ line.

There are a number of methods to introduce the exogenous DNA into the germ line (e.g., introduction into the germ or somatic cells) of a mammal. One method is by microinjection of a the gene construct into the pronucleus of an early stage embryo (e.g., before the four-cell stage) (Wagner, et al., *Proc. Natl. Acad. Sci. USA* 78:5016 (1981); Brinster, et al., *Proc Natl Acad Sci USA* 82:4438 (1985)). The detailed procedure to produce such transgenic mice has been described (see e.g., Hogan, et al., *Manipulating the Mouse Embryo*, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY (1986); US Patent No. 5,175,383 (1992)). This procedure has also been adapted for other mammalian species (e.g., Hammer, et al., *Nature* 315:680 (1985); Murray, et al., *Reprod. Fert. Devl.* 1:147 (1989); Pursel, et al., *Vet. Immunol. Histopath.* 17:303

(1987); Rexroad, et al., J. Reprod. Fert. 41 (suppl):119 (1990); Rexroad, et al., Molec. Reprod. Devl. 1:164 (1989); Simons, et al., BioTechnology 6:179 (1988); Vize, et al., J. Cell. Sci. 90:295 (1988); and Wagner, J. Cell. Biochem. 13B (suppl):164 (1989).

Another method for producing germ-line transgenic mammals is through the use of embryonic stem cells. The gene construct may be introduced into embryonic stem cells by homologous recombination (Thomas, et al., Cell 51:503 (1987); Capecchi, Science 244:1288 (1989); Joyner, et al., Nature 338: 153 (1989)). A suitable construct may also be introduced into the embryonic stem cells by DNA-mediated transfection, such as electroporation (Ausubel, et al., Current Protocols in Molecular Biology, John Wiley & Sons (1987)). Detailed procedures for culturing embryonic stem cells (e.g. ESD-3, ATCC# CCL-1934, ES-E14TG-2a, ATCC# CCL-1821, American Type Culture Collection, Rockville, MD) and the methods of making transgenic mammals from embryonic stem cells can be found in Teratocarcinomas and Embryonic Stem Cells, A Practical Approach, ed. E.J. Robertson (IRL Press, 1987).

In the above methods for the generation of a germ-line transgenic mammals, the construct may be introduced as a linear construct, as a circular plasmid, or as a vector which may be incorporated and inherited as a transgene integrated into the host genome. The transgene may also be constructed so as to permit it to be inherited as an extrachromosomal plasmid (Gassmann, M. et al., *Proc. Natl. Acad. Sci. USA* 92:1292 (1995)).

### Human Factor VIII

hFVIII is encoded by a 186 kilobase (kb) gene, with the coding region distributed among 26 exons (Gitchier et al., *Nature*, 312:326-330, (1984)). Transcription of the gene and splicing of the resulting primary transcript results in an mRNA of approximately 9 kb which encodes a primary translation product containing 2351 amino acids (aa), including a 19 aa signal peptide. Excluding the signal peptide, the 2332 aa protein has a domain structure which can be represented as NH<sub>2</sub>-A1-A2-B-A3-C1-C2-COOH, with a predicted molecular mass of 265 kilodaltons (kD). Glycosylation of this protein results in a product with a molecular mass of approximately 330 kD as determined by SDS-PAGE. In plasma, hFVIII is a heterodimeric protein consisting of a heavy chain that ranges in size from 90 kD to 200 kD in a metal ion complex with an 80 kD light chain. The heterodimeric complex is further stabilized by interactions with vWF. The heavy chain is comprised of domains A1-A2-B and the light chain is

comprised of domains A3-C1-C2 (Figure 2). Protease cleavage sites in the B-domain account for the size variation of the heavy chain, with the 90 kD species containing no B-domain sequences and the 200 kD species containing a complete or nearly complete B-domain. The B-domain has no known function and it is fully removed upon hFVIII activation by thrombin.

Human Factor VIII expression plasmids, plasmids pXF8.186 (Figure 3), pXF8.61 (Figure 4), pXF8.38 (Fig. 11) and pXF8.224 (Fig. 13) are described below. The hFVIII expression construct plasmid pXF8.186, was developed based on detailed optimization studies which resulted in high level expression of a functional hFVIII. Given the extremely large size of the hFVIII gene and the need to transfer the entire coding region into cells, cDNA expression plasmids were developed for the production of stably transfected clonal cell strains. It has proven difficult to achieve high level expression of hFVIII using the wild-type 9 kb cDNA. Three potential reasons for the poor expression are as follows. First, the wild-type cDNA encodes the 909 aa, heavily glycosylated B-domain which is transiently attached to the heavy chain and has no known function (Figure 1). Removal of the region encoding the B-domain from hFVIII expression constructs leads to greatly improved expression of a functional protein. Analysis of hFVIII derivatives lacking the B-domain has demonstrated that hFVIII function is not adversely affected and that such molecules have biochemical, immunologic, and in vivo functional properties which are very similar to the wild-type protein. Two different BDD hFVIII expression constructs have been developed, which encode proteins with different amino acid sequences flanking the deletion. Plasmid pXF8.186 contains a complete deletion of the B-domain (amino acids 741-1648 of the wild-type mature protein sequence), with the sequence Arg-Arg-Arg-Arg (RRRR) inserted at the heavy chain-light chain junction (Figure 1). This results in a string of five consecutive arginine residues (RRRRR or 5R) at the heavy chain-light chain junction, which comprises a recognition site for an intracellular protease of the PACE/furin class, and was predicted to promote cleavage to produce the correct heavy and light chains. Plasmid pXF8.61 also contains a complete deletion of the B-domain with a synthetic XhoI site at the junction. This linker results in the presence of the dipeptide sequence Leu-Glu (LE) at the heavy chain-light chain junction in the two forms of BDD hFVIII, the expressed proteins are referred to herein as 5R and LE BDD hFVIII.

The second feature which has been reported to adversely affect hFVIII expression in transfected cells relates to the observation that one or more regions of the coding region have

been identified which effectively function to block transcription of the cDNA sequence. The inventors have now discovered that the negative influence of the sequence elements can be reduced or eliminated by altering the entire coding sequence. To this end, a completely synthetic B-domain deleted hFVIII cDNA was prepared as described in greater detail below. Silent base changes were made in all codons which did not correspond to the triplet sequence most frequently found for that amino acid in highly expressed human proteins, and such codons were converted to the codon sequence most frequently found in humans for the corresponding amino acid. The resulting coding sequence has a total of 1094 of 4335 base pairs which differ from the wild-type sequence, yet it encodes a protein with the wild-type hFVIII sequence (with the exception of the deletion of the B-domain). 25.2% of the bases were changed, and the GC content of the sequence increased from 44% to 64%. This sequence-altered BDD hFVIII cDNA is expressed at least 5.3-fold more efficiently than a non-altered control construct.

The third feature which was optimized to improve hFVIII expression was the intron-exon structure of the expression construct. The cDNA is, by definition, devoid of introns. While this reduces the size of the expression construct, it has been shown that introns can have strong positive effects on gene expression when added to cDNA expression constructs. The 5' untranslated region of the human beta-actin gene, which contains a complete, functional intron was incorporated into the BDD hFVIII expression constructs pXF8.61 and pXF8.186.

The fourth feature which can adversely affect hFVIII expression is the stability of the Factor VIII mRNA. The stability of the message can affect the steady-state level of the Factor VIII mRNA, and influence gene expression. Specific sequences within Factor VIII can be altered so as to increase the stability of the mRNA, e.g., the removal of AURE from the 3' UTR can result in a more stable Factor VIII mRNA. The data presented below show that coding sequence re-engineering has general utility for the improvement of expression of mammalian and non-mammalian eukaryotic genes in mammalian cells. The results obtained here with human Factor VIII suggest that systemic codon optimization (with disregard to CpG content) provides a fruitful strategy for improving the expression in mammalian cells of a wide variety of eukaryotic genes.

A synthetic nucleic acid sequence which directs the synthesis of an optimized message of the invention can be made, e.g., by any of the methods described herein. The methods described below are advantageous for making optimized messages for the following reasons:

1) they allow for production of a highly optimized protein, e.g., a protein having at least 94 to 100% of codons as common codons, especially for proteins larger than 90 amino acids in length. The final product can be 100% optimized, i.e., every single nucleotide is as chosen, without the need to introduce undesirable alterations every 100 - 300 bp. A gene can be synthesized with 100% optimized codons, or it can be synthesized with 100% the codons that are desired. Additional DNA sequence elements can be introduced or avoided without any limitations imposed by the need to introduce restriction enzyme sites. Such sequence elements could include:

- Transcriptional signals, such as enhancers or silencers.
- Splicing signals, for example avoiding cryptic splice sites in a cDNA, or optimizing the splice site context in an intron-containing gene. Adding an intron to a cDNA may aid expression and allows the introduction of transcriptional signals within the gene.
- Instability signals – the creation or avoidance of sequences that direct mRNA breakdown.
- Secondary structure - the creation or avoidance of secondary structures in the mRNA that may affect mRNA stability, transcriptional termination, or translation.
- Translational signals - Codon choice. A gene can be synthesized with 100% optimal codons, or the codon bias for any amino acid can be altered without restriction to make gene expression sensitive to the concentration of an amino-acyl-tRNA, whose concentration may vary with growth or metabolic conditions.

In each case, the goal may be to increase or decrease expression to bring expression under a particular form of regulation.

2) they improve accuracy of the synthetic sequence because they avoid PCR amplification which introduces errors into the amplified sequence; and

3) they reduce the cost of making the synthetic sequence of the invention.

The synthetic nucleic acid sequence which direct the synthesis of the optimized messages of the invention can be prepared, e.g., by using the strategy which is outlined in greater detail below.

### Strategy for building a sequence

The initial step is to devise a cloning protocol.

A sequence file containing 100% the desired DNA sequence is generated. This sequence is analyzed for restriction sites, including fusion sites.

Fusion sites are, in order of preference:

A) Sequences resulting from the ligation of two complementary overhangs normally generated by available restriction enzymes, e.g.,

$\text{SalI/XhoI} = \begin{array}{l} \text{G}^{\wedge}\text{TCGAG} \\ \text{CAGCT}^{\wedge}\text{C} \end{array}$

or  $\text{BspDI/BstBI} = \begin{array}{l} \text{AT}^{\wedge}\text{CGAA} \\ \text{TAGC}^{\wedge}\text{TT} \end{array}$

or  $\text{BstBI/AccI} = \begin{array}{l} \text{TT}^{\wedge}\text{CGAC} \\ \text{AAGC}^{\wedge}\text{TG.} \end{array}$

B) Sequences resulting from the ligation of two overhangs generated by partially filling-in the overhangs of available restriction enzymes, e.g.,

$\text{XhoI(+TC)/BamHI(+GA)} = \begin{array}{l} \text{CTC}^{\wedge}\text{GATCC.} \\ \text{GAGCT}^{\wedge}\text{AGG} \end{array}$

C) Sequences resulting from the blunt ligation of two blunt ends normally generated by available restriction enzymes, e.g.,

$\text{EheI/SmaI} = \begin{array}{l} \text{GGC}^{\wedge}\text{GGG} \\ \text{CCG}^{\wedge}\text{CCC.} \end{array}$

D) Sequences resulting from the blunt ligation of two blunt ends, where one or both blunt ends have been generated by filling in an overhang, e.g.,

$\text{BamHI(+GATC)/SmaI} = \begin{array}{l} \text{GGATC}^{\wedge}\text{GGG} \\ \text{CCTAG}^{\wedge}\text{CCC} \end{array}$



The filling-in of a 5' overhang generated by a restriction enzyme is performed using a DNA polymerase, for example the Klenow fragment of DNA Polymerase I. If the overhang is to be filled in completely, then all four nucleotides, dATP, dCTP, dGTP, and dTTP, are included in the reaction.. If the overhang is to be only partially filled in, then the requisite nucleotides are omitted from the reaction, In item (B) above, the XhoI-digested DNA would be filled in by Klenow in the presence of dCTP and dTTP and by omitting dATP and dGTP. An order of cloning steps is determined that allows the use of sites about 150-500 bp apart. Note that a fragment must lack the recognition sequence for an enzyme, only if that enzyme is used to clone the fragment. For example, the strategy for the construction of the "desired" Factor VIII coding sequence can use ApaLI in a number of different places, because of the order of assembly of the fragments - ApaLI is not used in any of the later cloning steps.

If there is a region where no useful sites are available, then a sequence-independent strategy can be used: fragments are cloned into a DNA construct that contain recognition sequences for restriction enzymes that cleave outside of their recognition sequence,

e.g. BseRI = GAGGAGNNNNNNNNNN^ (SEQ ID NO:5)

CTCCTCNNNNNNNNNN^NN (SEQ ID NO:6)

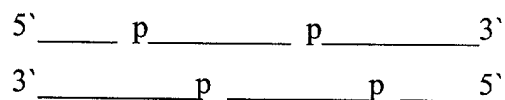
DNA construct cloning site gene fragment

The recognition sequence of the enzyme used to clone the fragment will be removed when the fragment is released by digestion with, e.g. BseRI, leaving a fragment consisting of 100% of the desired sequence, which can then be ligated to a similarly generated adjacent gene fragment.

The next step is to synthesize initial restriction fragments.

The synthesis of the initial restriction fragments can be achieved in a number of ways, including, but not limited to:

1. Chemical synthesis of the entire fragment.
2. Synthesize two oligonucleotides that are complementary at their 3' ends, anneal them, and use DNA polymerase Klenow fragment, or equivalent, to extend, giving a double-stranded fragment.
3. Synthesize a number of smaller oligonucleotides, kinase those oligo's that have internal 5' ends, anneal all oligo's and ligate, viz.



Techniques 2 and 3 can be used in subsequent steps to join smaller fragments to each other. PCR can be used to increase the quantity of material for cloning, but it may lead to an increase in the number of mutations. If an error-free fragment is not obtained, then site-directed mutagenesis can be used to correct the best isolate. This is followed by concatenation of error-free fragments and sequencing of junctions to confirm their precision.

### Use

The synthetic nucleic acid sequences of the invention are useful for expressing a protein normally expressed in a mammalian cell, or in cell culture (e.g. for commercial production of human proteins such as GH, tPA, GLP-1, EPO,  $\alpha$ -galactosidase,  $\beta$ -glucocereamidase,  $\alpha$ -iduronidase;  $\alpha$ -L-iduronidase, glucosamine-N-sulfatase, alpha-N-acetylglucosaminidase, acetylcoenzyme A: $\alpha$ -glucosmainide-N-acetyltransferase, N-acetylglucosamine-6-sulfatase, N-acetylglucosamine-6-sulfatase,  $\beta$ -galactosidase, N-acetylgalactosamine-6-sulfatase,  $\beta$ -glucuronidase. Factor VIII, and Factor IX). The synthetic nucleic acid sequences of the invention are also useful for gene therapy. For example, a synthetic nucleic acid sequence encoding a selected protein can be introduced directly, e.g., via non-viral cell transfection or via a vector in to a cell, e.g., a transformed or a non-transformed cell, which can express the protein to create a cell which can be administered to a patient in need of the protein. Such cell-based gene therapy techniques are described in greater detail in co-pending US applications: USSN 08/334,797; USSN 08/231,439; USSN 08/334,455; and USSN 08/928,881, which are hereby expressly incorporated by reference in their entirety.

## Examples

### Construction of pXF8.61

The fourteen gene fragments of the B-domain-deleted-FVIII optimized cDNA listed in Table 2 and shown in Figure 5 (Fragment A-Fragment N) were made as follows. 92 oligonucleotides were made by oligonucleotide synthesis on an ABI 391 synthesizer (Perkin Elmer). The 92 oligonucleotides are listed in Table 3. Figure 5 shows how these 92 oligonucleotides anneal to form the fourteen gene fragments of Table 2. For each strand of each gene fragment, the first oligonucleotide (i.e. the most 5') was manufactured with a 5'-hydroxyl terminus, and the subsequent oligonucleotides were manufactured as 5'-phosphorylated to allow the ligation of adjacent annealed oligonucleotides. For gene fragments A,B,C,F,G,J,K,L,M and N, six oligonucleotides were annealed, ligated, digested with EcoRI and HindIII and cloned into pUC18 digested with EcoRI and HindIII. For gene fragments D, E, H and I, eight oligonucleotides were annealed, ligated, digested with EcoRI and HindIII and cloned into pUC18 digested with EcoRI and HindIII. This procedure generated fourteen different plasmids--pAM1A through pAM1N.

**Table 2**

Fragment	5' end		3' end		Note
A	NheI	1	ApaI	279	
B	ApaI	279	PmII	544	
C	PmII	544	PmII	829	
D	PmII	829	BglII(/BamHI)	1172	BamHI site 3' to seq
E	(BglII/)Bam HI	1172	BglII	1583	
F	BglII	1583	KpnI	1817	
G	KpnI	1817	BamHI	2126	
H	BamHI	2126	PmII	2491	
I	PmII	2491	KpnI	3170	ΔBstEII 2661-2955

J	BstEII	2661	BstEII	2955	
K	KpnI	3170	ApaI	3482	
L	ApaI	3482	SmaI/(EcoRV)	3772	
M	(SmaI)/EcoR V	3772	BstEII	4062	
N	BstEII	4062	SmaI	4348	

In Table 2 the restriction site positions are numbered by the first base of the palindrome; numbering begins at the NheI site.

**Table 3**

Oligo' Name	Oligo' Length	Oligonucleotide Sequence
AM1Af1	118	GTAGAATTCGTAGGCTAGCATGCAGATCGAGCTGAGCACCTGCTTCTCCTGTGCCTGCTGCGCTTCTGCTTCAGCGCCACCCGCCGCTACTACCTGGGCGCCGTGGAGCTGAGCTGG (SEQ ID NO:7)
AM1Af2	104	GACTACATGCAGAGCGACCTGGGCGAGCTGCCCCTGGACGCCCGCTTCCCCCCCCGCGTGCCCAAGAGCTTCCCTTCAACACCAGCGTGGTGTACAAGAAGAC (SEQ ID NO: 8)
AM1Af3	88	CCTGTTTCGTGGAGTTCACCGACCACCTGTTCAACATCGCCAAGCCCCGCCCCCTGGATGGGCCTGCTGGGCCCTACAAGCTTTAC (SEQ ID NO: 9)
AM1Ar1	119	GTAAAGCTTGTAGGGGCCAGCAGGCCCATCCAGGGGGGGCGGGGCTTGGCGATGTTGAACAGGTGGTCGGTGAAGTCCACGAACAGGGTCTTCTTGACACCACGCTGGTGTGAAGG (SEQ ID NO: 10)
AM1Ar2	107	GGAAGCTCTTGGGCACGCGGGGGGGGAAGCGGGCGTCCACGGGCAGCTCGCCAGGTCTCTGCATGTAGTCCAGCTCAGCTCCACGGCGCCAGGTAGTAGCGG (SEQ ID NO: 11)
AM1Ar3	84	CGGGTGGCGCTGAAGCAGAAGCGCAGCAGGCACAGGAAGAAGCAGGTGCTCAGCTCGATCTGCATGCTAGCCTACGAATTCTAC (SEQ ID NO: 12)
AM1Bf1	115	GTAGAATTCGTAGGGGCCCCACCATCCAGGCCGAGGTGTACGACACCGTGGTGATCACCC TGAAGAACATGGCCAGCCACCCCGTGAGCCTGCACGCCGTGGGCGTGAGCTACTG (SEQ ID NO: 13)
AM1Bf2	103	GAAGGCCAGCGAGGGCGCCGAGTACGACGACCAGACCAGCCAGCGCGAGAAGGAGGACGACAAGGTGTTCCCGGCGGCAGCCACACCTACGTGTGGCAGGTG (SEQ ID NO: 14)
AM1Bf3	79	CTGAAGGAGAACGGCCCCATGGCCAGCGACCCCTGTGCCTGACCTACAGCTACCTGAGC CACGTGCTACAAGCTTTAC (SEQ ID NO: 15)
AM1Br1	107	GTAAAGCTTGTAGCACGTGGCTCAGGTAGCTGTAGGTCAGGCACAGGGGGTTCGCTGGCC ATGGGGCCGTTCTCCTTCAGCACCTGCCACACGTAGGTGTGGCTGCCG (SEQ ID NO: 16)
AM1Br2	101	CCGGGGAACACCTTGTCTCCTCCTTCTCGCGCTGGCTGGTCTGGTCGTCTGCTACTCGGCGC CCTCGCTGGCCTTCCAGTAGCTCACGCCACGGCGTGCAG (SEQ ID NO: 17)
AM1Br3	89	GCTCACGGGGTGGCTGGCCATGTTCTTCAGGGTGATCACCACGGTGTCTGTACACCTCGGC CTGGATGGTGGGGCCCTACGAATTCTAC (SEQ ID NO: 18)
AM1Cf1	122	GTAGAATTCGTAGCCACGTGGACCTGGTGAAGGACCTGAACAGCGGCCTGATCGGCGCC CTGCTGGTGTGCCGAGGGCAGCCTGGCCAAGGAGAAGACCCAGACCTGCACAAGTTC ATC (SEQ ID NO: 19)
AM1Cf2	110	CTGCTGTTCCGCGTGTTCGACGAGGGCAAGAGCTGGCACAGCGAGACCAAGAAGAGCCT GATGCAGGACCGCGACGCCGCCAGCGCCCGCGCTGGCCAAGATGCACAC (SEQ ID NO: 20)

		20)
AM1Cf3	86	CGTGAACGGCTACGTGAACCGCAGCCTGCCCCGGCCTGATCGGCTGCCACCGCAAGAGCG TGTAAGTGGCACGTGCTACAAGCTTTAC (SEQ ID NO: 21)
AM1Cr1	108	GTAAGCTTGTAGCACGTGCCAGTACACGCTCTTGCGGTGGCAGCCGATCAGGCCGGGCA GGCTGCGGTTACGTAGCCGTTACGGTGTGCATCTTGGGCCAGGCGC (SEQ ID NO: 22)
AM1Cr2	110	GGGCGCTGGCGGCGTCGCGGTCCTGCATCAGGCTGTTCTTGGTCTCGCTGTGCCAGCTCTT GCCCTCGTCGAACACGGCGAACAGCAGGATGAACCTGTGCAGGGTCTGG (SEQ ID NO: 23)
AM1Cr3	100	GTCTTCTCCTTGGCCAGGCTGCCCTCGCGGCACACCAGCAGGGCGCCGATCAGGCCGCTG TTCAGGTCCTTACCAGGTCCACGTGGCTACGAATTCTAC (SEQ ID NO: 24)
AM1Df1	99	GTAAGATTCGTAGCACGTGATCGGCATGGGCACACCCCGAGGTGCACAGCATCTTCCT GGAGGGCCACACCTTCCTGGTGCACACACCGCCAGGC (SEQ ID NO: 25)
AM1Df2	100	CAGCCTGGAGATCAGCCCCATCCTTCTGACCGCCAGACCCTGCTGATGGACCTGGG CCAGTTCCTGCTGTTCTGCCACATCAGCAGCCACCAGCAC (SEQ ID NO: 26)
AM1Df3	101	GACGGCATGGAGGCTACGTGAAGGTGGACAGCTGCCCCGAGGAGCCCCAGCTGCGCAT GAAGAACAACGAGGAGGCCGAGGACTACGACGACGACCTGAC (SEQ ID NO: 27)
AM1Df4	84	CGACAGCGAGATGGACGTGGTGCCTTCGACGACGACAACAGCCCCAGCTTCATCCAGA TCTCTACGGATCCTACAAGCTTTAC (SEQ ID NO: 28)
AM1Dr1	109	GTAAGCTTGTAGGATCCGTAGAGATCTGGATGAAGCTGGGGCTGTTGTCGTCGTCGAAG CGCACCACGTCCATCTCGCTGTCGGTCAGGTCGTCGTCGTAGTCCTCGG (SEQ ID NO: 29)
AM1Dr2	101	CCTCTCGTTGTTCTTCATGCGCAGCTGGGGCTCCTCGGGGCAGCTGTCCACCTTCACGTA GGCTCCATGCCGTCGTGCTGGTGGCTGCTGATGTGGCAG (SEQ ID NO: 30)
AM1Dr3	102	AACAGCAGGAAGTGGCCCAGGTCCATCAGCAGGGTCTGGGCGGTCAGGAAGGTGATGGG GCTGATCTCCAGGCTGGCCTGGCGGTGGTTGCGCACCAGGAAG (SEQ ID NO: 31)
AM1Dr4	72	GTGTGGCCCTCCAGGAAGATGCTGTGCACCTCGGGGGTGGTGGCCATGCCGATCACGTGC TACGAATTCTAC (SEQ ID NO: 32)
AM1Ef1	122	GTAAGATTCGTAGGGATCCGCAGCGTGGCCAAGAAGCACCCCAAGACCTGGGTGCACTA CATCGCCGCCGAGGAGGAGGACTGGGACTACGCCCCCTGGTGTGCCCCCGACGACCGC AG (SEQ ID NO: 33)
AM1Ef2	120	CTACAAGAGCCAGTACCTGAACAACGGCCCCCAGCGCATCGGCCGCAAGTACAAGAAGG TGCGCTTCATGGCCTACACCGACGAGACCTTCAAGACCCGCGAGGCCATCCAGCACGAGAG (SEQ ID NO: 34)
AM1Ef3	115	CGGCATCCTGGGCCCCCTGCTGTACGGCGAGGTGGGCGACACCTGCTGATCATCTTCAA GAACCAGGCCAGCCGCCCTACAACATCTACCCCCACGGCATCACCGACGTGCGC (SEQ ID NO: 35)
AM1Ef4	86	CCCCTGTACAGCCGCCGCTGCCCAAGGGCGTGAAGCACCTGAAGGACTTCCCCATCCTG CCCGGCGAGATCTCTACAAGCTTTAC (SEQ ID NO: 36)
AM1Er1	109	GTAAGCTTGTAGAGATCTCGCCGGGCAGGATGGGGAAGTCTTCAGGTGCTTCACGCCC TTGGGCAGGCGGCGGCTGTACAGGGGGCGCACGTCGGTGATGCCGTGGG (SEQ ID NO: 37)
AM1Er2	114	GGTAGATGTTGTAGGGGCGGCTGGCCTGGTTCTTGAAGATGATCAGCAGGGTGTGCCCCA CCTCGCCGTACAGCAGGGGGCCAGGATGCCGCTCTCGTGCTGGATGGCCTCGC (SEQ ID NO: 38)
AM1Er3	121	GGGTCTTGAAGGTCTCGTCGGTGTAGGCCATGAAGCGCACCTTCTTGTACTTGGCGCCGA TGCGCTGGGGGCCGTTGTTTCAAGTACTGGCTCTTGTAGCTGCGGTCGTCGGGGGCCAGCAC (SEQ ID NO: 39)
AM1Er4	99	CAGGGGGGCGTAGTCCCAGTCCTCCTCCTCGGCGGCGATGTAGTGCACCCAGGTCTTGGG GTGCTTCTTGGCCACGCTGCGGATCCCTACGAATTCTAC (SEQ ID NO: 40)
AM1Ff1	102	GTAAGATTCGTAGAGATCTTCAAGTACAAGTGGACCGTGACCGTGAGGACGGCCCCAC CAAGAGCGACCCCGCTGCTGACCCGCTACTACAGCAGCTTC (SEQ ID NO: 41)
AM1Ff2	103	GTGAACATGGAGCGCGACCTGGCCAGCGGCCTGATCGGCCCCCTGCTGATCTGCTACAAG GAGAGCGTGACCGAGCGCGGAACAGATCATGAGCGACAAGC (SEQ ID NO: 42)
AM1Ff3	61	GCAACGTGATCCTGTTTACGCGTGTTCGACGAGAACCGCAGCTGGTACCCTACAAGCTTTA C (SEQ ID NO: 43)
AM1Fr1	87	GTAAGCTTGTAGGGTACCAGCTGCGGTTCTCGTCGAACACGCTGAACAGGATCACGTTG CGCTTGTGCTCATGATCTGGTTGCCG (SEQ ID NO: 44)

AM1Fr2	101	CGCTGGTCCACGCTCTCCTTGTAGCAGATCAGCAGGGGGCCGATCAGGCCGCTGGCCAGGTCGCGCTCCATGTTACGAAGCTGCTGTAGTAGCGGGTCAG (SEQ ID NO: 45)
AM1Fr3	78	GCAGCGGGGGTCGCTCTTGGTGGGGCCGTCCTCCACGGTCACGGTCCACTTGTACTTGAA GATCTCTACGAATTCTAC (SEQ ID NO: 46)
AM1Gf1	120	GTAGAATTCGTAGGGTACCTGACCGAGAACATCCAGCGCTTCCTGCCCAACCCCGCCGGC GTGCAGCTGGAGGACCCCGAGTTCAGGCCAGCAACATCATGCACAGCATCAACGGCTAC (SEQ ID NO: 47)
AM1Gf2	126	GTGTTTCGACAGCCTGCAGCTGAGCGTGTGCCTGCACGAGGTGGCCTACTGGTACATCCTG AGCATCGGCGCCAGACCGACTTCCTGAGCGTGTCTTCAGCGGCTACACCTTCAAGCACA AGATG (SEQ ID NO: 48)
AM1Gf3	95	GTGTACGAGGACACCTGACCCTGTTCCCTTCAGCGGCGAGACCGTGTTCATGAGCATG GAGAACCCCGGCCTGTGGATCCCTACAAGCTTTAC (SEQ ID NO: 49)
AM1Gr1	119	GTAAGCTTGTAGGGATCCACAGGCCGGGGTTCCTCATGCTCATGAACACGGTCTCGCCG CTGAAGGGGAACAGGGTCAGGGTGTCTCGTACACCATCTTGTGCTTGAAGGTGTAGCC (SEQ ID NO: 50)
AM1Gr2	124	GCTGAAGAACACGCTCAGGAAGTCGGTCTGGGCGCCGATGCTCAGGATGTACCAGTAGG CCACCTCGTGCAGGCACACGCTCAGCTGCAGGCTGTGGAACACGTAGCCGTTGATGCTGTG CATG (SEQ ID NO: 51)
AM1Gr3	98	ATGTTGCTGGCCTGGAACCTCGGGGTCTCCAGCTGCACGCCGGCGGGGTTGGGCAGGAA GCGCTGGATGTTCTCGGTACAGTACCCTACGAATTCTAC (SEQ ID NO: 52)
AM1Hf1	111	GTAGAATTCGTAGGGATCCTGGGCTGCCACAACAGCGACTTCGCAACCGCGGCATGACC GCCCTGCTGAAGGTGAGCAGCTGCGACAAGAACACCGGCGACTACTACGAG (SEQ ID NO: 53)
AM1Hf2	102	GACAGCTACGAGGACATCAGCGCCTACCTGCTGAGCAAGAACAACGCCATCGAGCCCCG CCTGGAGGAGATCACCCGCACCACCCTGCAGAGCGACCAGGAG (SEQ ID NO: 54)
AM1Hf3	105	GAGATCGACTACGACGACACCATCAGCGTGGAGATGAAGAAGGAGGACTTCGACATCTA CGACGAGGACGAGAACCAGAGCCCCGCAGCTTCAGAGAAGAAGACC (SEQ ID NO: 55)
AM1Hf4	79	CGCCACTACTTCATCGCCGCCGTGGAGCGCCTGTGGGACTACGGCATGAGCAGCAGCCCC CACGTGCTACAAGCTTTAC (SEQ ID NO: 56)
AM1Hr1	101	GTAAGCTTGTAGCACGTGGGGGCTGCTGCTCATGCCGTAGTCCACAGGCGCTCCACGG CGGCGATGAAGTAGTGGCGGGTCTTCTTCTGGAAGCTGCGG (SEQ ID NO: 57)
AM1Hr2	105	GGGCTCTGGTTCTCGTCCTCGTCGTAGATGTGGAAGTCTCCTTCTTCATCTCCACGCTGA TGGTGTGCTCGTAGTCGATCTCCTCCTGGTCTGCTCTGCAGGGTG (SEQ ID NO: 58)
AM1Hr3	108	GTGCGGGTGATCTCCTCCAGGCGGGGCTCGATGGCGTTGTTCTTGTCTCAGCAGGTAGGCG CTGATGTCCTCGTAGCTGTCTCCTCGTAGTAGTCGCCGGTGTCTTGTGCG (SEQ ID NO: 59)
AM1Hr4	83	CAGCTGCTCACCTTCAGCAGGGCGGTATGCCGCGGTTGCGGAAGTCGCTGTTGTGGCAG CCCAGGATCCCTACGAATTCTAC (SEQ ID NO: 60)
AM1If1	115	GTAGAATTCGTAGCACGTGCTGCGCAACCGCGCCCAGAGCGGCAGCGTGCCCCAGTTCA AGAAGGTGGTGTTCAGGAGTTCACCGACGGCAGCTTACCCAGCCCCTGTACCGC (SEQ ID NO: 61)
AM1If2	111	GGCGAGCTGAACGAGCACCTGGGCCTGCTGGGCCCTACATCCGCGCCGAGGTGGAGGA CAACATCATGGTGACCGTGCAGGAGTTCGCCCTGTTCTTACCATCTTCGAC (SEQ ID NO: 62)
AM1If3	106	GAGACCAAGAGCTGGTACTTCACCGAGAACATGGAGCGCAACTGCCGCGCCCCCTGCAA CATCCAGATGGAGGACCCACCTTCAAGGAGAACTACCGCTTCCACG (SEQ ID NO: 63)
AM1If4	85	CCATCAACGGCTACATCATGGACACCCTGCCCGCCTGGTGATGGCCAGGACCAGCGCA TCCGCTGGTACCCTACAAGCTTTAC (SEQ ID NO: 64)
AM1Ir1	115	GTAAGCTTGTAGGGTACCAGCGGATGCGCTGGTCTGGGCCATCACCAGGCCGGGCGAG GGTGTCCATGATGTAGCCGTTGATGGCGTGGAAGCGGTAGTTCTCCTTGAAGGTGG (SEQ ID NO: 65)
AM1Ir2	99	GGTCTCCATCTGGATGTTGCAGGGGGCGCGGCAGTTGCGCTCCATGTTCTCGGTGAAGT ACCAGCTCTTGGTCTCGTCGAAGATGGTGAAGAACAGGG (SEQ ID NO: 66)
AM1Ir3	110	CGAACTCCTGCAGGTCACCATGATGTTGTCTCCACCTCGGCGCGGATGTAGGGGCCCA GCAGGCCAGGTGCTCGTTACGCTCGCCGCGGTACAGGGGCTGGGTGAAG (SEQ ID NO: 67)

AM1Ir4	93	CTGCCGTCGGTGAACCTCCTGGAACACCACCTTCTTGAACCTGGGGCACGCTGCCGCTCTGG GCGCGGTTGCGCAGCACGTGCTACGAATTCTAC (SEQ ID NO: 68)
AM1Jf1	116	GTAGAATTCGTAGGGTGACCTTCCGCAACCAGGCCAGCCGCCCTACAGCTTCTACAGCA GCCTGATCAGCTACGAGGAGGACCAGCGCCAGGGCGCCGAGCCCCGCAAGAACTTC (SEQ ID NO: 69)
AM1Jf2	120	GTGAAGCCCAACGAGACCAAGACCTACTTCTGGAAGGTGCAGCACCATATGGCCCCAC CAAGGACGAGTTCGACTGCAAGGCCTGGGCCTACTTCAGCGACGTGGACCTGGAGAAGGA C (SEQ ID NO: 70)
AM1Jf3	91	GTGCACAGCGGCCTGATCGGCCCCCTGCTGGTGTGCCACACCAACACCCTGAACCCCGCC CACGGCCGCCAGGTGACCCTACAAGCTTTAC (SEQ ID NO: 71)
AM1Jr1	113	GTAAGCTTGTAGGGTGACCTGGCGGCCGTGGGCGGGGTTAGGGGTGTTGGTGTGGCACA CCAGCAGGGGGCCGATCAGGCCGCTGTGCACGTCCTTCTCCAGGTCCACGTCG (SEQ ID NO: 72)
AM1Jr2	121	CTGAAGTAGGCCAGGCCTTGCACTGCAACTCGTCCTTGGTGGGGGCCATGTGGTGTCTGC ACCTTCCAGAAGTAGGTCTTGGTCTCGTTGGGCTTCACGAAGTCTTGCGGGGCTCGGCGC (SEQ ID NO: 73)
AM1Jr3	93	CCTGGCGCTGGTCTCTCGTAGCTGATCAGGCTGCTGTAGAAGCTGTAGGGGCGGCTGG CCTGGTTGCGGAAGGTACCCCTACGAATTCTAC (SEQ ID NO: 74)
AM1Kf1	120	GTAGAATTCGTAGGGTGACCTGCTGAGCATGGGCAGCAACGAGAACATCCACAGCATCCA CTTCAGCGGCCACGTGTTACCGTGCGCAAGAAGGAGGAGTACAAGATGGCCCTGTACAAC (SEQ ID NO: 75)
AM1Kf2	122	CTGTACCCCGGCGTGTTCGAGACCGTGGAGATGCTGCCAGCAAGGCCGGCATCTGGCGC GTGGAGTGCCTGATCGGCGAGCACCTGCACGCCGGCATGAGCACCTGTTCCTGGTGTACA G (SEQ ID NO: 76)
AM1Kf3	102	CAACAAGTGCAGACCCCCCTGGGCATGGCCAGCGGCCACATCCGCGACTTCCAGATCAC CGCCAGCGGCCAGTACGCCAGTGGGCCCTACAAGCTTTAC (SEQ ID NO: 77)
AM1Kr1	123	GTAAGCTTGTAGGGGGCCACTGGCCGTACTGGCCGCTGGCGGTGATCTGGAAGTCGCGG ATGTGGCCGCTGGCCATGCCAGGGGGTCTGGCACTTGTGTGCTGTACACCAGGAACAGGG TG (SEQ ID NO: 78)
AM1Kr2	125	CTCATGCCGCGTGCAGGTGCTCGCCGATCAGGCACTCCACGCGCCAGATGCCGGCCTTG CTGGGCAGCATCTCCACGGTCTCGAACACGCCGGGGTACAGGTTGTACAGGGCCATCTTGT ACTC (SEQ ID NO: 79)
AM1Kr3	96	CTCCTTCTTGCGCACGGTGAACACGTGGCCGCTGAAGTGGATGCTGTGGATGTTCTCGTT GCTGCCCATGCTCAGCAGGTACCCTACGAATTCTAC (SEQ ID NO: 80)
AM1Lf1	120	GTAGAATTCGTAGGGGGCCCCAAGCTGGCCCGCCTGCACTACAGCGGCAGCATCAACGC CTGGAGACCAAGGAGCCCTTCAGCTGGATCAAGGTGGACCTGCTGGCCCCATGATCATC (SEQ ID NO: 81)
AM1Lf2	116	CACGGCATCAAGACCCAGGGCGCCCGCCAGAAGTTCAGCAGCCTGTACATCAGCCAGTT CATCATCATGTACAGCCTGGACGCAAGAAGTGGCAGACCTACCGCGGCAACAGCAC (SEQ ID NO: 82)
AM1Lf3	86	CGGCACCCTGATGGTGTCTTCGGCAACGTGGACAGCAGCGGCATCAAGCACAACATCTT CAACCCCCCGGGCTACAAGCTTTAC (SEQ ID NO: 83)
AM1Lr1	110	GTAAGCTTGTAGCCCGGGGGGTTGAAGATGTTGTGCTTGTATGCCGCTGCTGTCCACGT TGCCGAAGAACACCATCAGGGTGCCGGTGTGTTGCCGCGGTAGGTCTGC (SEQ ID NO: 84)
AM1Lr2	113	CACTTCTTGCCGTCCAGGCTGTACATGATGATGAACTGGCTGATGTACAGGCTGCTGAAC TTCTGGCGGGCGCCCTGGGTCTTGTATGCCGTGGATGATCATGGGGGCCAGCAG (SEQ ID NO: 85)
AM1Lr3	99	GTCCACCTTGATCCAGCTGAAGGGCTCCTTGGTGTCTCCAGGCGTTGATGCTGCCGCTGTA GTGCAGGCGGGCCAGCTTGGGGGCCCTACGAATTCTAC (SEQ ID NO: 86)
AM1Mf1	122	GTAGAATTCGTAGGATATCATCGCCCGCTACATCCGCCTGCACCCCACTACAGCAT CCGCAGCACCTGCGCATGGAGCTGATGGGCTGCGACCTGAACAGCTGCAGCATGCCCTG G (SEQ ID NO: 87)
AM1Mf2	112	GCATGGAGAGCAAGGCCATCAGCGACGCCAGATCACCGCCAGCAGCTACTTCACCAAC ATGTTGCCACCTGGAGCCCCAGCAAGGCCCGCTGCACCTGACGGGCCGAG (SEQ ID

		NO: 88)
AM1Mf3	89	CAACGCCTGGCGCCCCCAGGTGAACAACCCCAAGGAGTGGCTGCAGGTGGACTTCCAGA AGACCATGAAGGTGACCCTACAAGCTTTAC (SEQ ID NO: 89)
AM1Mr1	112	GTAAAGCTTGTAGGGTCACCTTCATGGTCTTCTGGAAGTCCACCTGCAGCCACTCCTTGG GGTTGTTACCTGGGGGCGCCAGGCGTTGCTGCGGCCCTGCAGGTGCAGGCG (SEQ ID NO: 90)
AM1Mr2	114	GGCCTTGCTGGGGCTCCAGGTGGCGAACATGTTGGTGAAGTAGCTGCTGGCGGTGATCTG GGCGTCGCTGATGGCCTTGCTCTCCATGCCAGGGGCATGCTGCAGCTGTTTAC (SEQ ID NO: 91)
AM1Mr3	97	GTCGCAGCCCATCAGCTCCATGCGCAGGGTGCTGCGGATGCTGTAGTGGGTGGGGTGCAG GCGGATGTAGCGGGCGATGATATCCTACGAATTCTAC (SEQ ID NO: 92)
AM1Nf1	122	GTAGAATTCGTAGGGTGACCGGCGTGACCACCCAGGGCGTGAAGAGCCTGCTGACCAGC ATGTACGTGAAGGAGTTCCTGATCAGCAGCAGCCAGGACGGCCACCAGTGGACCCTGTTCT TC (SEQ ID NO: 93)
AM1Nf2	104	CAGAACGGCAAGGTGAAGGTGTTCCAGGGCAACCAGGACAGCTTACCCCCGTGGTGAA CAGCCTGGACCCCCCTGCTGACCCGCTACCTGCGCATCCACCC (SEQ ID NO: 94)
AM1Nf3	92	CCAGAGCTGGGTGCACCAGATCGCCCTGCGCATGGAGGTGCTGGGCTGCGAGGCCCAGG ACCTGTACTAGCTGCCCCGGGCTACAAGCTTTAC (SEQ ID NO: 95)
AM1Nr1	118	GTAAAGCTTGTAGCCCGGGCAGCTAGTACAGGTCTGGGCCTCGCAGCCAGCACCTCCA TGCGCAGGGCGATCTGGTGCACCCAGCTCTGGGGGTGGATGCGCAGGTAGCGGGTCAG (SEQ ID NO: 96)
AM1Nr2	100	CAGGGGGGGGTCCAGGCTGTTACCCAGGGGGTGAAGCTGTCCTGGTTGCCCTGGAACA CCTTCACCTTGCCGTTCTGGAAGAACAGGGTCCACTGGTGG (SEQ ID NO: 97)
AM1Nr3	100	CCGTCCTGGCTGCTGCTGATCAGGAATCCTTCACGTACATGCTGGTGCAGCAGGCTCTTCA CGCCCTGGGTGGTCACGCCGGTCACCCTACGAATTCTAC (SEQ ID NO: 98)

As noted in Table 2 and shown in Figure 5, fragment D was constructed with a BamHI restriction site placed between the BglII site and the HindIII site at the 3' end of the fragment. Fragment I was constructed to carry the DNA from PmlI (2491) to BstEII (2661) followed immediately by the DNA from BstEII (2955) to KpnI (3170), so that the insertion of the BstEII fragment from pAMJ into the BstEII site of pAMI in the correct orientation will generate the desired sequences from 2491 to 3170. Plasmid pAM1B was digested with ApaI and HindIII and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1A digested with ApaI and HindIII, generating plasmid pAM1AB. Plasmid pAM1D was digested with PmlI and HindIII and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1AB digested with PmlI and HindIII, generating plasmid pAM1ABD. Plasmid pAM1C was digested with PmlI and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1ABD digested with PmlI, generating plasmid pAM1ABCD, insert orientation was confirmed by the appearance of a diagnostic 111bp fragment when digested with MscI. Plasmid pAM1F was digested with BglII and HindIII and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1E digested with BglII and HindIII, generating



plasmid pAM1EF. Plasmid pAM1G was digested with KpnI and HindIII and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1EF digested with KpnI and HindIII, generating plasmid pAM1EFG. Plasmid pAM1J was digested with BstEII and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1I digested with BstEII, generating plasmid pAM1IJ; orientation was confirmed by the appearance of a diagnostic 465bp fragment when digested with EcoRI and EagI. Plasmid pAM1IJ was digested with PmlI and HindIII and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1H digested with PmlI and HindIII, generating plasmid pAM1HIJ. Plasmid pAM1M was digested with EcoRI and BstEII and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1N digested with EcoRI and BstEII, generating plasmid pAM1MN. Plasmid pAM1L was digested with EcoRI and SmaI and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1MN digested with EcoRI and EcoRV, generating plasmid pAM1LMN. Plasmid pAM1LMN was digested with ApaI and HindIII and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1K digested with ApaI and HindIII, generating plasmid pAM1KLMN. Plasmid pAM1EFG was digested with BamHI and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1ABCD digested with BamHI and BglII, generating plasmid pAM1ABCDEFG; orientation was confirmed by the appearance of a diagnostic 552bp fragment when digested with BglII and HindIII. Plasmid pAM1KLMN was digested with KpnI and HindIII and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1HIJ digested with KpnI and HindIII, generating plasmid pAM1HIJKLMN. Plasmid pAM1HIJKLMN was digested with BamHI and HindIII and the insert was purified by agarose gel electrophoresis and inserted into plasmid pAM1ABCDEFG digested with BamHI and HindIII, generating plasmid pAM1-1. These cloning steps are depicted in Figure 6. Figure 7 shows the DNA sequence of the insert contained in pAM1-1 (SEQ ID NO:1). This insert can be cloned into any suitable expression vector as a NheI-SmaI fragment to generate an expression construct. pXF8.61 (Fig. 4), pXF8.38 (Fig. 11) and pXF8.224 (Fig. 13) are examples of such a construct.

### **Construction of pXF8.186**

The "LE" version of the B-domain-deleted-FVIII optimized cDNA contained in pAM1-1 was modified by replacing the Leu-Glu dipeptide (2284-2289) at the junction of the heavy and light

chains with four Arginine residues, making a total of five consecutive Arginine residues (SEQ ID NO:2). This was achieved as follows. The six oligonucleotides shown in Table 4 were annealed, ligated, digested with EcoRI and HindIII and cloned into pUC18 digested with EcoRI and HindIII, generating the plasmid pAM8B. Figure 8 shows how these oligonucleotides anneal to form the requisite DNA sequence. pAM8B was digested with BamHI and BstXI and the 230bp insert was purified by agarose gel electrophoresis and used to replace the BamHI(2126)-BstXI(2352) fragment of the "LE" version (See Figure 7). Figure 9 shows the sequence of the resulting cDNA (SEQ ID NO:2). This "5Arg" version of the B-domain-deleted-FVIII optimized cDNA can be cloned into any suitable expression vector as a NheI-SmaI fragment to generate an expression construct. pXF8.186 (Figure 3) is an example of such a construct.

**Table 4**

OLIGO' NAME	OLIGO' LENGTH	OLIGONUCLEOTIDE SEQUENCE
AM8F1	140	GTAGAATTCGGATCCTGGGCTGCCACAACAGCGACTT CCGCAACCGCGGCATGACCGCCCTGCTGAAGGTGAGC AGCTGCGACAAGAACACCGGCGACTACTACGAGGAC AGCTACGAGGACATCAGCGCCTACCTGCTG (SEQ ID NO:99)
AM8BF2	57	AGCAAGAACAACGCCATCGAGCCCCGCAGGCGCAGG CGCGAGATCACCCGCACCACC (SEQ ID NO:100)
AM8F4	58	CTGCAGAGCGACCAGGAGGAGATCGACTACGACGAC ACCATCAGCGTGGAAGCTTTAC (SEQ ID NO:101)
AM8R1	79	GTAAAGCTTCCACGCTGATGGTGTCTGTCGTAGTCGAT CTCTCCTGGTCGCTCTGCAGGGTGGTGCGGGTGATCT CGCG (SEQ ID NO:102)
AM8BR2	57	CCTGCGCCTGCGGGGCTCGATGGCGTTGTTCTTGCTCA GCAGGTAGGCGCTGATGTC (SEQ ID NO:103)
AM8BR4	119	CTCGTAGCTGTCCTCGTAGTAGTCGCCGGTGTTCCTTGT CGCAGCTGCTCACCTTCAGCAGGGCGGTGATGCCGCG GTTGCGGAAGTCGCTGTTGTGGCAGCCCAGGATCCGA ATTCTAC (SEQ ID NO:104)

### **Construction of pXF8.36**

The construct for expression of human Factor VIII, pXF8.36 (Fig. 10) is an 11.1 kilobase circular DNA plasmid which contains the following elements: A cytomegalovirus immediate early I gene (CMV) 5' flanking region comprised of a promoter sequence, a 5' untranslated sequence (5'UTS) and first intron sequence for initiation of transcription of the Factor VIII cDNA. The CMV region is next fused with a wild-type B domain-deleted Factor VIII cDNA sequence. The Factor VIII cDNA sequence is fused, at the 3' end, with a 0.3 kb fragment of the human growth hormone 3' untranslated sequence. A transcription termination signal and 3' untranslated sequence (3' UTS) of the human growth hormone gene is used to ensure processing of the message immediately following the stop codon. A selectable marker gene (the bacterial neomycin phosphotransferase (*neo*) gene) is inserted downstream of the Factor VIII cDNA to allow selection for stably transfected mammalian cells using the neomycin analog G418. Expression of the neo gene is under the control of the simian virus 40 (SV40) early promoter. The pUC 19-based amplicon carrying the pBR322-derived- $\beta$ -lactamase (*amp*) and origin of replication (*ori*) allows for the uptake, selection and propagation of the plasmid in E coli K-12 strains. This region was derived from the plasmid pBSII SK+.

### **Construction of pXF8.38**

The construct for expression of human Factor VIII, pXF8.38 (Fig. 11) is an 11.1 kilobase circular DNA plasmid which contains the following elements: A cytomegalovirus immediate early I gene (CMV) 5' flanking region comprised of a promoter sequence, 5' untranslated sequence (5'UTS) and first intron sequence for initiation of transcription of the Factor VIII cDNA. The CMV region is next fused with a synthetic, optimally configured B domain-deleted Factor VIII cDNA sequence. The Factor VIII cDNA sequence is fused, at the 3' end, with a 0.3 kb fragment of the human growth hormone 3' untranslated sequence. A transcription termination signal and 3' untranslated sequence (3' UTS) of the human growth hormone gene is used to ensure processing of the message immediately following the stop codon. A selectable marker gene (the bacterial neomycin phosphotransferase (*neo*) gene) to allow selection for stably transfected mammalian cells using the neomycin analog G418 is inserted downstream of the Factor VIII cDNA. Expression of the neo gene is under the control of the simian virus 40

(SV40) early promoter. The pUC 19-based amplicon carrying the pBR322-derived  $\beta$ -lactamase (amp) and origin of replication (ori) allows for the uptake, selection and propagation of the plasmid in E coli K-12 strains. This region was derived from the plasmid pBSII SK+.

### **pXF8.269 Construct**

The construct for expression of human Factor VIII (Fig. 12), pXF8.269, is a 14.8 kilobase (kb) circular DNA plasmid which contains the following elements: A human collagen (I)  $\alpha$  2 promoter which contains 0.17 kb of 5' untranslated sequence (5'UTS), Aldolase A gene 5' untranslated sequence (5'UTS) and first intron sequence for initiation of transcription of the Factor VIII cDNA. The aldolase intron region is next fused with a synthetic, wild-type B domain-deleted Factor VIII cDNA sequence. A transcription termination signal and 3' untranslated sequence (3'UTS) of the human growth hormone gene to ensure processing of the message immediately following the stop codon. A selectable marker gene (the bacterial neomycin phosphotransferase (neo) gene) to allow selection for stably transfected mammalian cells using the neomycin analog G418 is inserted downstream of the Factor VIII cDNA.. The expression of the neo gene is under the control of the SV40 promoter The pUC 19-based amplicon carrying the pBR322-derived  $\beta$ -lactamase (amp) and origin of replication (ori) allows for the uptake, selection and propagation of the plasmid in E coli K-12 strains. This region was derived from the plasmid pBSII SK+.

### **pXF8.224 Construct**

The construct for expression of human Factor VIII, pXF8.224 (Fig 13), is a 14.8 kilobase (kb) circular DNA plasmid which contains the following elements: A human collagen (I)  $\alpha$  2 promoter which contains 0.17 kb of 5' untranslated sequence (5'UTS), aldolase A gene 5' untranslated sequence (5'UTS) and first intron sequence for initiation of transcription of the Factor VIII cDNA. The aldolase intron region is next fused with a synthetic, optimally configured B domain-deleted Factor VIII cDNA sequence. A transcription termination signal and 3' untranslated sequence (3'UTS) of the human growth hormone gene is used to ensure processing of the message immediately following the stop codon. A selectable marker gene (the bacterial neomycin phosphotransferase (neo) gene) to allow selection for stably transfected

mammalian cells using the neomycin analog G418 is inserted downstream of the Factor VIII cDNA. The expression of the neo gene is under the control of the SV40 promoter. The pUC 19-based amplicon carrying the pBR322-derived- $\beta$ -lactamase (*amp*) and origin of replication (*ori*) allows for the uptake, selection and propagation of the plasmid in E coli K-12 strains. This region was derived from the plasmid pBSII SK+.

### **Clotting Assay**

A clotting assay based on an activated partial thromboplastin time (aPTT) (Proctor, et al., *Am. J. Clin. Path.*, 36:212-219, (1961)) was performed to analyze the biological activity of the BDD hFVIII molecules expressed by constructs in which BDD-FVIII coding region was optimized.

### **Biological activity as analyzed using the clotting Assay**

The results of the aPTT-based clotting assay are presented in Table 5, below. Specific activity of the hFVIII preparations is presented as aPTT units per milligram hFVIII protein as determined by ELISA. Both of the human fibroblast-derived BDD hFVIII molecules (5R and LE) have high specific activity when measured the aPTT clotting assay. These specific activities have been determined to be up to 2- to 3-fold higher than those determined for CHO cell-derived full-length FVIII (as shown in Table 5). An average of multiple determinations of specific activities for various partially purified preparations of 5R and LE BDD hFVIII also shows consistently higher values for the BDD hFVIII molecules (11,622 Units/mg for 5R BDD hFVIII, and 14,561 Units/mg for LE BDD hFVIII as compared to 7097 Units/mg for full-length CHO cell-derived FVIII). An increased rate and/or extent of thrombin activation has been observed for various~BDD hFVIII molecules, possibly due to an effect of the B-domain to protect the heavy and light chains from thrombin cleavage and activation (Eaton et al., *Biochemistry*, 25:8343-8347, (1986), Meulien et al., *Protein Engineering*, 2:301-306, (1988)).

Table 5. Specific Activities of Various hFVIII Proteins

<b>hFVII Product</b>	<b>Concen- tration by ELISA (mg/mL)</b>	<b>aPTT Activity (aPTT U/mL)</b>	<b>Specific Activity (aPTT U/mg)</b>
<b>5R BDD hFVIII</b>	0.050	1306	26,120
<b>LEBDD HFVIII</b>	0.124	2908	23,452
<b>Full-length (CHO- derived) FVIII</b>	0.158	1454	9202

**Assay for Human Factor VIII in Transfected Cell Culture Supernatants.**

Samples of cell culture, supernatants having cells transfected with wild-type, or optimized human BDD-human Factor VIII were assayed for human Factor VIII (hFVIII) content by using an enzyme-linked immunosorbent assay (ELISA). This assay is based on the use of two non-crossreacting monoclonal antibodies (mAb) in conjunction with samples consisting of cell culture media collected from the supernatants of transfected human fibroblast cells.

Methods of transfection and identification of positively transfected cells are described in the U.S. Patent No. 5,641,670, which is incorporated herein by reference

**Table 6**

Plasmid	Promoter / 5' Untranslated sequence	Factor VIII cDNA Composition	Mean (FVIII mU / 10 <sup>6</sup> Cells / 24 hr.)	Maximum (FVIII mU / 10 <sup>6</sup> Cells / 24 hr.)	Number of Strains	Fold increase
pXF8.36	CMV IE1	Wild Type	567	2557	38	--
pXF8.38	CMV IE1	Optimal Configuration	5403	17106	24	9.5X
pXF8.269	Collagen I $\alpha$ 2 / Aldolase Intron	Wild Type	382	1227	18	--
pXF8.224	Collagen I $\alpha$ 2 / Aldolase Intron	Optimal Configuration	2022	11930	218	5.3X

ELISA units based on standard curves prepared from pooled normal plasma.

All patents and other references cited herein are hereby incorporated by reference.

### Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1 1. A synthetic nucleic acid sequence which encodes a protein wherein at least one non-  
2 common codon or less-common codon has been replaced by a common codon, and having one or  
3 more of the following properties:

4 (i) the synthetic nucleic acid sequence comprises a continuous stretch of at least  
5 90 codons all of which are common codons;

6 (ii) the synthetic nucleic acid sequence comprises a continuous stretch of common  
7 codons, which continuous stretch includes at least 33% or more of the codons in the synthetic  
8 nucleic acid sequence; or

9 (iii) wherein at least 94% or more of the codons in the sequence encoding the  
10 protein are common codons and wherein the synthetic nucleic acid sequence encodes a protein of  
11 at least about 90 amino acids in length.

1 2. The synthetic nucleic acid sequence of claim 1, wherein said synthetic nucleic acid  
2 sequence encodes a protein wherein at least one non-common codon or less-common codon has  
3 been replaced by a common codon, and wherein the synthetic nucleic acid sequence comprises a  
4 continuous stretch of at least 90 codons all of which are common codons.

1 3. The synthetic nucleic acid sequence of claim 1, wherein said synthetic nucleic acid  
2 sequence encodes a protein wherein at least one non-common codon or less-common codon has  
3 been replaced by a common codon, and wherein the synthetic nucleic acid sequence comprises a  
4 continuous stretch of common codons, which continuous stretch includes at least 33% or more of  
5 the codons in the synthetic nucleic acid sequence.

1 4. The synthetic nucleic acid sequence of claim 1, wherein said synthetic nucleic acid  
2 sequence encodes a protein wherein at least one non-common codon or less-common codon has  
3 been replaced by a common codon, and wherein at least 94% or more of the codons in the  
4 sequence encoding the protein are common codons and wherein the synthetic nucleic acid  
5 sequence encodes a protein of at least about 90 amino acids in length.





1 14. The nucleic acid of claim 1, wherein at least 98% of the codons in the synthetic  
2 nucleic acid sequence are common codons.

1 15. A synthetic nucleic acid sequence which encodes Factor VIII, wherein at least one  
2 non-common codon or less-common codon has been replaced by a common codon and wherein  
3 the synthetic nucleic acid has one or more of the following properties: it has a continuous stretch  
4 of at least 90 codons all of which are common codons; it has a continuous stretch of common  
5 codons which comprise at least 33% of the codons of the synthetic nucleic acid sequence; at least  
6 94% or more of the codons in the sequence encoding the protein are common codons and the  
7 synthetic nucleic acid sequence encodes a protein of at least about 90 amino acids in length; it is  
8 at least 80 base pairs in length.

1 16. The synthetic nucleic acid sequence of claim 15 where the factor VIII protein has  
2 one or more of the following characteristics:

3 a) the B domain is deleted (BDD factor VIII);

4 b) it has a recognition site for an intracellular protease of the PACE/furin class;

5 or

6 c) it is inserted into a non-transformed cell.

1 17. The synthetic nucleic acid sequence of claim 15, wherein the number of non-  
2 common or less- common codons replaced or remaining is less than 15.

1 18. The synthetic nucleic acid sequence of claim 15, wherein the number of non-  
2 common or less- common codons replaced or remaining, taken together, are equal or less than  
3 6% of the codons in the synthetic nucleic acid sequence.

1 19. The synthetic nucleic acid sequence of claim 15, wherein all non- common or less-  
2 common codons are replaced with common codons.

1 20. The synthetic nucleic acid sequence of claim 15, wherein all non- common and less-  
2 common codons are replaced with common codons.



1 29. The synthetic nucleic acid sequence of claim 24, wherein all non- common and less-  
2 common codons are replaced with common codons.

1 30. The synthetic nucleic acid sequence of claim 24, wherein at least 96% of the codons  
2 in the synthetic nucleic acid sequence are common codons.

1 31. The synthetic nucleic acid sequence of claim 24, wherein at least 98% of the codons  
2 in the synthetic nucleic acid sequence are common codons.

1 32. The synthetic nucleic acid sequence of claim 24, wherein all of the codons are  
2 replaced with common codons.

1 33. A vector comprising the synthetic nucleic acid sequence of claim 1, 15, or 24.

1 34. A cell comprising the nucleic acid sequence of claim 1, 15, or 24.

1 35. A method for preparing a synthetic nucleic acid sequence which is at least 90 codons  
2 in length, comprising:

3 identifying a non-common codon and a less-common codon in a non-optimized  
4 gene sequence which encodes a protein; and

5 replacing at least 94% of the non-common and less-common codons with a  
6 common codon encoding the same amino acid as the replaced codon.

1 36. The method of claim 35, wherein at least 96% of the non-common and less-common  
2 codons are replaced with a common codon encoding the same amino acid as the replaced codon.

1 37. The method of claim 35, wherein at least 98% of the non-common and less-common  
2 codons are replaced with a common codon encoding the same amino acid as the replaced codon

1 38. The method of claim 35, wherein the nucleic acid sequence encodes a protein of at  
2 least about 105 or more codons in length.

1 39. A method for making a nucleic acid sequence which directs the synthesis of an  
2 optimized message of a protein of at least 90 amino acids comprising:

3 synthesizing at least two fragments of the nucleic acid sequence, wherein the two  
4 fragments encode adjoining portions of the protein and wherein both subunits are mRNA  
5 optimized; and

6 joining the two fragments such that a non-common codon is not created at a  
7 junction point, thereby making the mRNA optimized nucleic acid sequence.

1 40. The method of claim 39, wherein the two fragments are joined together such that a  
2 unique restriction endonuclease site is not created at the junction point.

1 41. The method of claim 39, wherein the two fragments are joined together such that a  
2 unique restriction site is created.

1 42. The method of claim 39, wherein three fragments of the nucleic acid sequence are  
2 synthesized.

1 43. The method of claim 39, wherein the synthetic nucleic acid sequence encodes a  
2 protein of 105 or more codons in length.

1 44. The method of claim 39, wherein 96% of the codons in the synthetic nucleic acid  
2 sequence are common codons.

1 45. The method of claim 39, wherein 98% of the codons in the synthetic nucleic acid  
2 sequence are common codons.

1 46. The method of claim 39, wherein all of the codons in the synthetic nucleic acid  
2 sequence are common codons.

1 47. The method of claim 39, wherein the number of codons which are not common  
2 codons is equal to or less than 15.

1 48. The method of claim 39, wherein each fragment is at least 30 codons in length.

49. A method of providing a subject with a protein or polypeptide, comprising:

- providing a synthetic nucleic acid sequence that can direct the synthesis of an optimized message for a protein or polypeptide;
- introducing the synthetic nucleic acid sequence into the subject; and
- allowing the subject to express the protein or polypeptide, thereby providing the subject with the protein.

50. The method of claim 49, wherein the synthetic nucleic acid is introduced into a cell.

51. The method of claim 50, wherein the cell can be an autologous, allogenic, or xenogeneic cell.

52. The method of claim 50 wherein the cell is a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells.

53. The method of claim 49, wherein the codon optimized synthetic nucleic acid sequence can be inserted into the cell *ex vivo* or *in vivo*.

54. The method of claim 49, wherein at least 94%, or all of the codons in the synthetic nucleic acid sequence are common codons.

55. The method of claim 49, wherein at least 96%, or all of the codons in the synthetic nucleic acid sequence are common codons.

56. The method of claim 49, wherein at least 98%, or all of the codons in the synthetic nucleic acid sequence are common codons.

57. The method of claim 49, wherein the number of codons which are not common codons is equal to or less than 15.

1 58. A method for preparing a synthetic nucleic acid sequence encoding a protein which  
2 is at least 90 codons in length, comprising identifying non-common codon and less-common  
3 codons in the non-optimized gene encoding the protein and replacing at least 94% or more of the  
4 non-common and less-common codons with a common codon encoding the same amino acid as  
5 the replaced codon.

1 59. A primary or secondary cell of vertebrate origin having an exogenous synthetic  
2 nucleic acid sequence which encodes a protein or a polypeptide wherein at least one non-  
3 common codon or less-common codon has been replaced by a common codon and wherein the  
4 synthetic nucleic acid has one or more of the following properties: it has a continuous stretch of  
5 at least 90 codons all of which are common codons; it has a continuous stretch of common  
6 codons which comprise at least 33% of the codons of the synthetic nucleic acid sequence; at least  
7 94% or more of the codons in the sequence encoding the protein are common codons and the  
8 synthetic nucleic acid sequence encodes a protein of at least about 90 amino acids in length; it is  
9 at least 80 base pairs in length and which is free of unique restriction endonuclease sites that  
10 would occur in the message optimized sequence; and

11 DNA sequences, sufficient for expression of the exogenous synthetic DNA in the  
12 transfected primary or secondary cell;

13 the primary or secondary cell capable of expressing the protein or polypeptide product.

1 60. The primary or secondary cell of claim 59, wherein the exogenous synthetic nucleic  
2 acid is transfected into the cell.

1 61. The primary or secondary cell of claim 59, wherein the exogenous synthetic nucleic  
2 acid sequence is stably integrated into its genome.

1 62. The primary or secondary cell of claim 59, wherein the exogenous synthetic nucleic  
2 acid is present in the cell in an episome.

63. The primary or secondary cell of claim 59, wherein the DNA sequence sufficient for  
expression of the exogenous synthetic nucleic acid is of non-viral origin.

[illegible]

	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2423	2424	2425	2426	2427	2428	2429	2430	2431	2432	2433	2434	2435	2436	2437	2438	2439	2440	2441	2442	2
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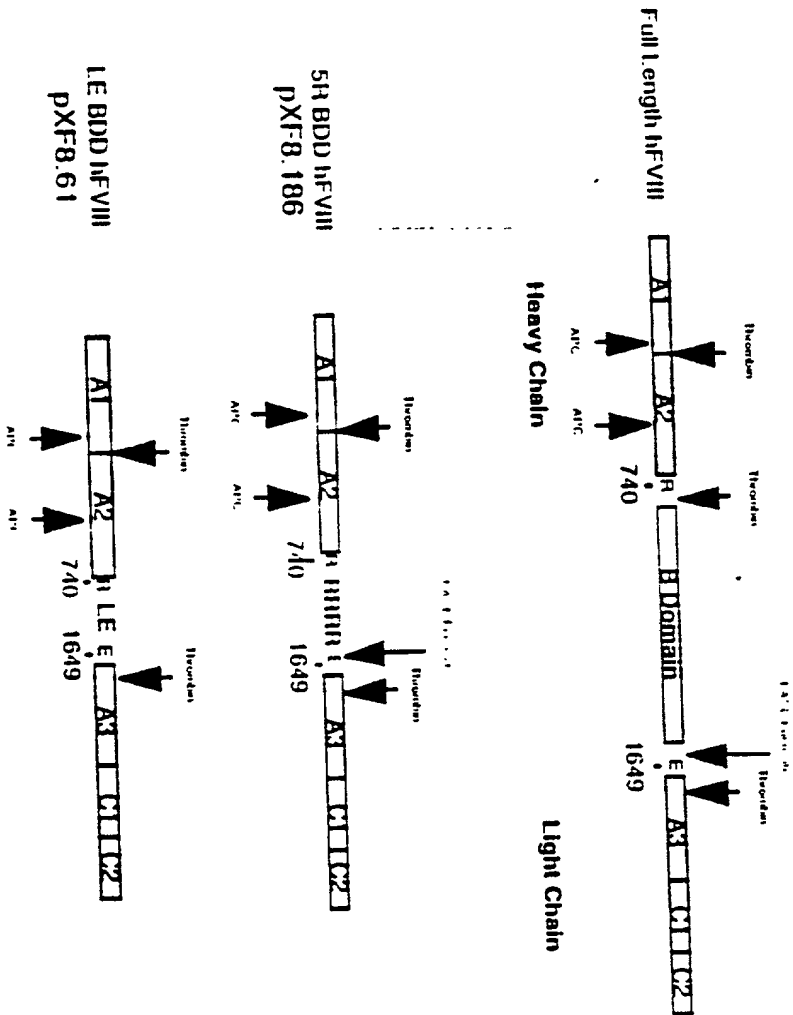
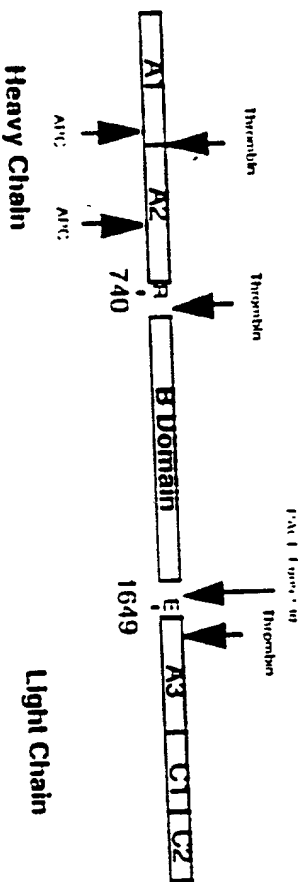


FIG. 1

03403605 03403605



Heterogeneity of hFVIII is due to proteolysis  
within the B-domain



FIG. 2

00407606 000000

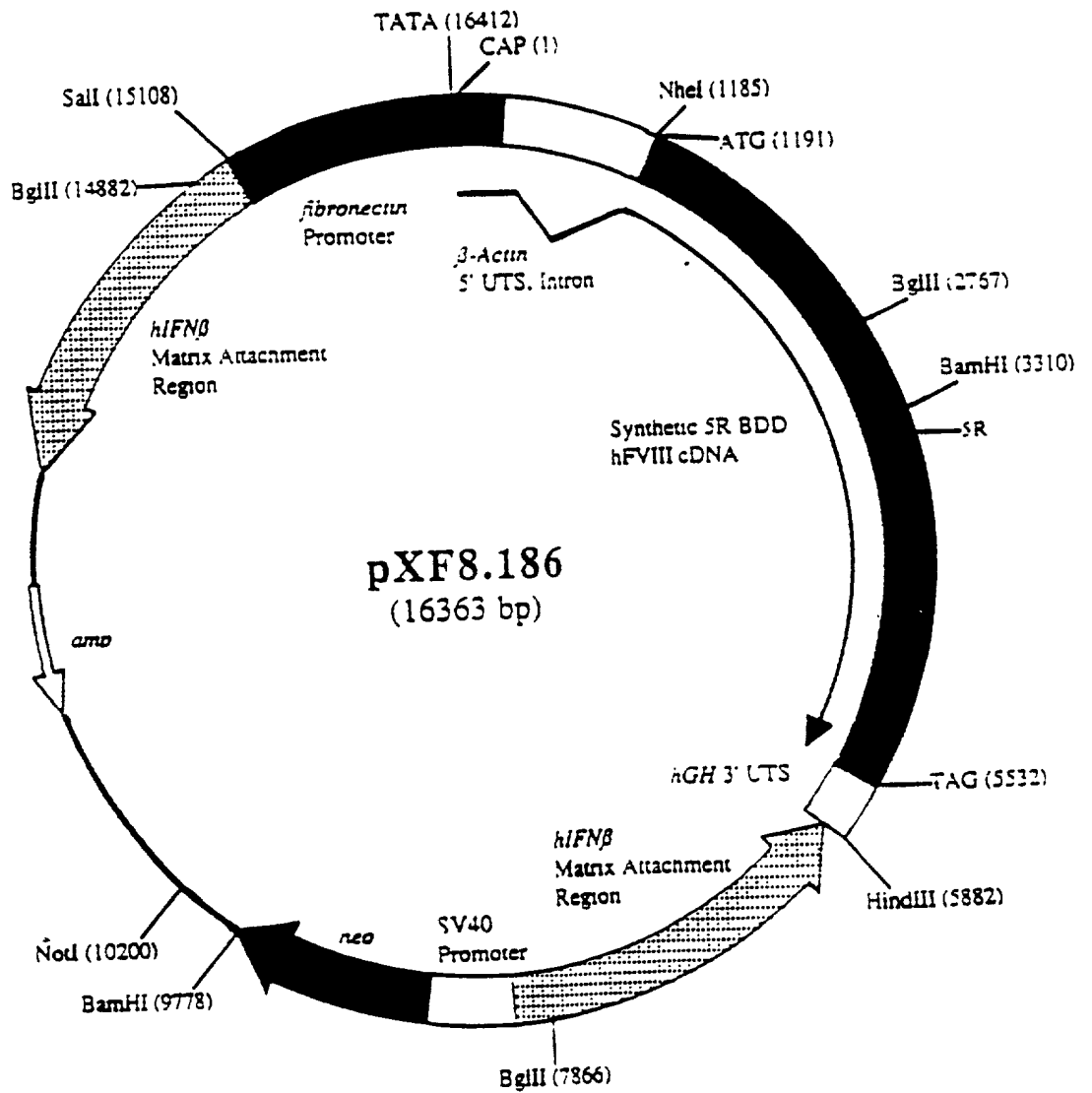


FIG. 3

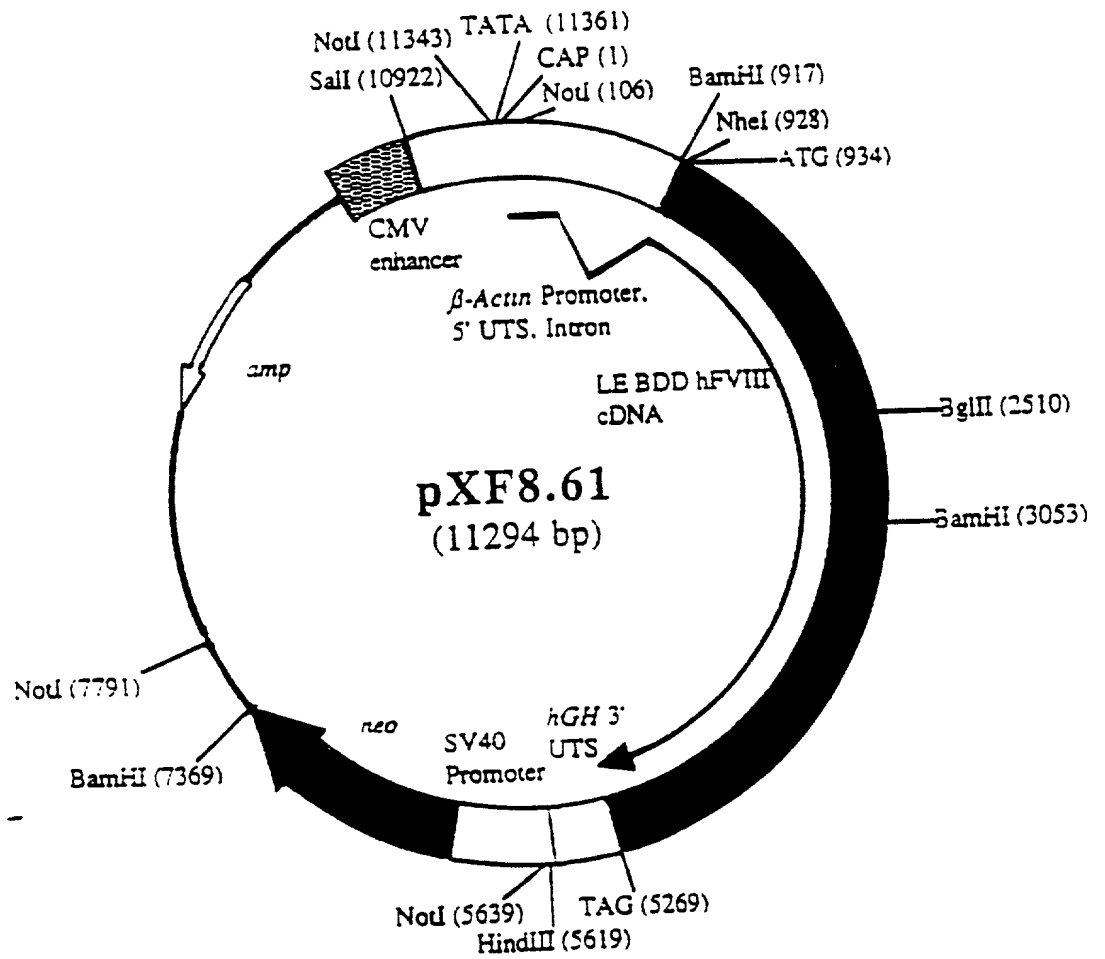


FIG. 4

# Fragment A

	EcoRI	NheI		AM1 A11
	GTAGAATTCTGCTAGCCTTAGCATCCAGATCCAGCCTGAGCACCCTGCTTCTCTGTGCTGCTGCGCTTCTGC			
	CATCTTAAAGCATCCGATCCGTTACGTTCTACCTCGACTCTGTGACGAGAGAGAGACGACGACGCGMAGACG		AM1 A13	
			3' OH 5' P	
	TTCAAGCGCCACCCCTCCGCTACCTACCTGCGCCCGCGTGGAGCTGAGCTGGG			
	AGTTCGCGGTGGCGGCGCATGATGGACCTCCCGGCACCTCGACTCGACCCTCATGTACGTCCTCCGCTGGA		AM1 A12	
	5' P 3' OH			
	GGCGGAGCTGCGCGTGGACGCCCCCTTCCCCCGCGGTGCCCCAGAGCTTCCCTCTTCAACACCAAGCT			
	CTCCGCTTGAAGGGGACCTTCGCGCCGAGGGGGGCGCACGGGTTCTTCCGAGG		AM1 A13	
	3' OH 5' P		5' P 3' OH	
	GGTGTACAGAGAGACCTGTGTTCGTGGAGTTCACCGACCACTGTTCAACATCGCCAAAGCCCCCCCC			
	CCACATGTTCTCTTGCGACAGCACTTCACAGTGGCTGTCGACAGTTCGAGCGGTTCGGGGGGGGGG		AM1 A11	
	CTGGATGGGCGCTGCTGGGCGCCCTTACAAAGCTTTAC		AM1 A11	
	GACCTTACCCCGACGACCCCGGCTCATGTTCGAAATG			

FIG. 5 (1 of 14)

# Fragment B

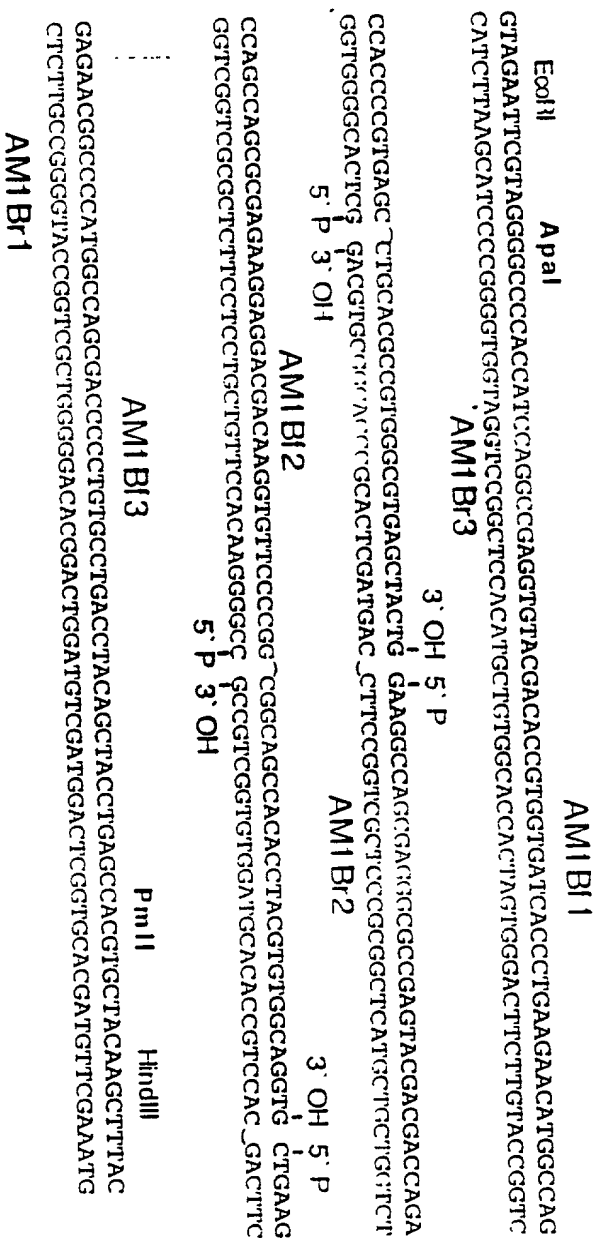


FIG. 5 (2 of 14)

# Fragment C

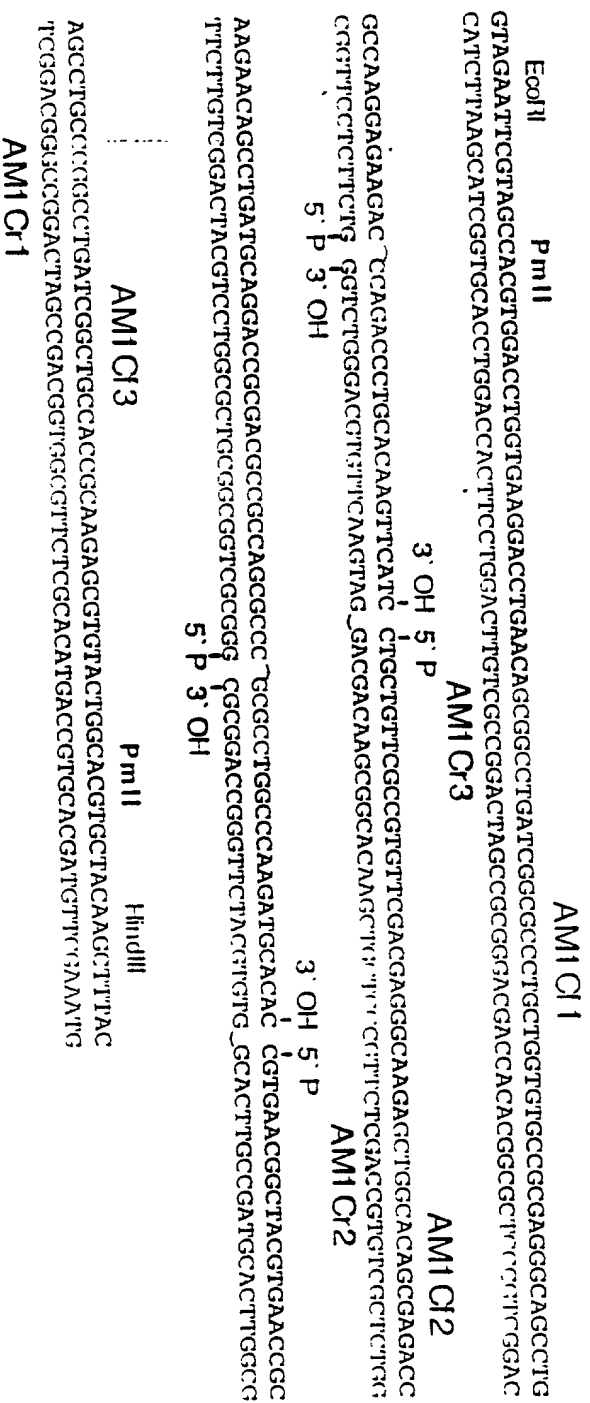


FIG. 5 (3 of 14)

## Fragment D

[illegible]

**FIG. 5 (4 of 14)**



# Fragment E

EcoRI      BamHI      AM1E1  
 GTAGAAATTCGTAGGGATCCGACGGCTGGCCAGAAGCACCCCAAGACCTGGGTTCACCTACATCGCCGCGAGGAGGAGGACTGGGACTACGC  
 CTATCTTAGCATCCCTAGGCGTCGCACCGGTTCTTCGTGGGGTTCTGGACCCACGTGATGTATCTTCGGCTTCTCTGACCCCTGATATCTG  
 AM1E4  
 3' OH 5' P  
 EAGI  
 CCCCCCTGTCGCTGCCCCCGACGACCGCAGCTACAGAGCCAGTACCTGAACAACGGCCCCCAGCGCATCTGCGCGCAAGTACAGAAGGT  
 GGGGAGCACGACCGGGGGCTGCTGGCGTCTGATGTTCTCGGTCAAGACTTGTTCGGGGGTCCGCGTAGCCGGCTTCATGTTCTTCCCA  
 5' P 3' OH  
 AM1E3  
 3' OH 5' P  
 Apal  
 GCGCTTCATGGGCTACACCGACGAGACTTCAAGACCCCTCCGAGGCATCCAGACGAGAGCGGCATCCTGGGCCCCCTGCTGACGGCGA  
 CCGGAAGTACCGGATGTGGCTGCTTGAAAGTTCTGGGCGCTCCGGTAGGTCGCTTCTCCGTAGGACCCGGGGAGACGACATGCTCT  
 5' P 3' OH  
 AM1E13  
 3' OH 5' P  
 GGTGGGCGACACCCCTGCTGATCATCTTCAAGAACCAGGGCCAGCGCGCCCTTACAACATCTACCTCCACGGCATCACCAGCGTGGCCTCCCT  
 CCAACCCGCTGTGGGACGACTAGTAGAAGTTCTTGTGTCGGTCCGGTCCGGGGATGTTGTAGATGAGGGTCCGCTTAGAGATTGTTGAAATG  
 5' P 3' OH  
 AM1E2  
 BglII      HindIII  
 GTCACGCCCGCCGCTGCCCAAGGGCGTGAAGCACCTGAAGACTTCCCATCTGCCCCGGCGAGATCTCTACAAAGCTTTAC  
 CTATCTCGGGCGGCGACGAGTTCCCGCACTTCTGTGACTTCTTGAAAGGGGTAGAACGGGCTTCTTAGAGATTGTTGAAATG  
 AM1E1  
 AM1E14

FIG. 5 (5 of 14)

# Fragment F

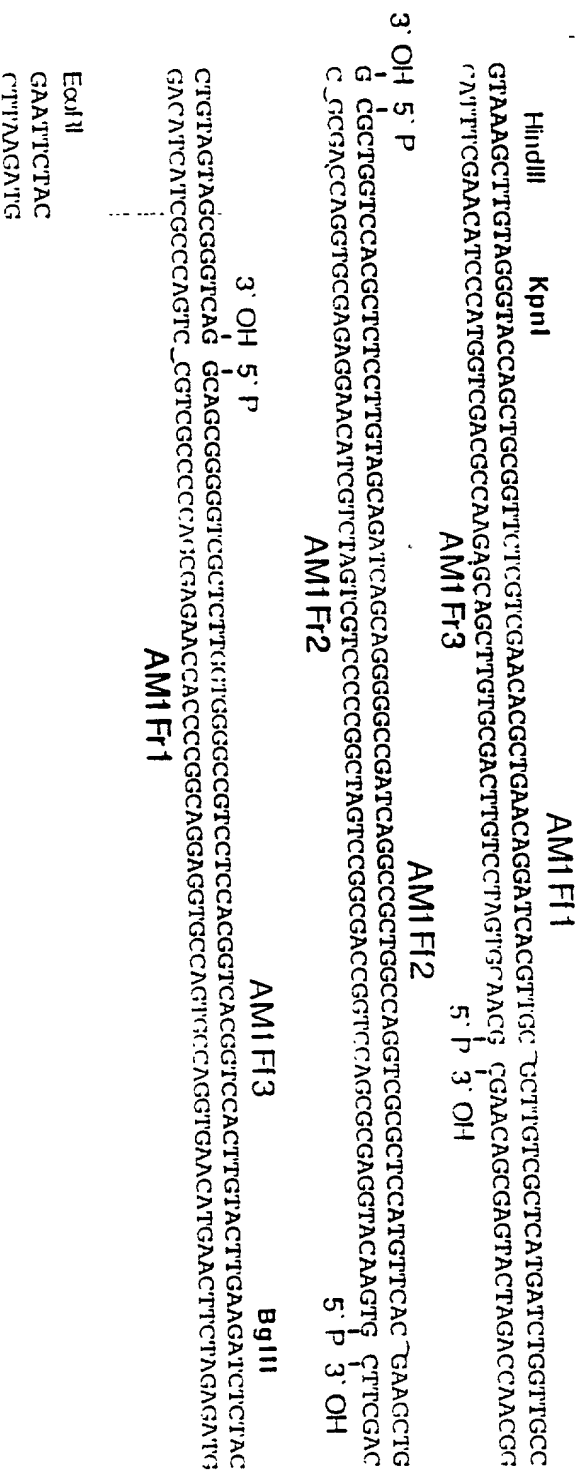


FIG. 5 (6 of 14)

# Fragment G

EcoRI	KpnI		AM1G11
GTAGATTCGTAGGGTACCTGACCGAGAACAATCCAGCGCTTCCTGCCCCACCCCGCGCGTGCAGCTGAGGACCCCGAGTTCCAGGCCAG			
CAATCTTAGCAATCCCATGGA	CTGGCTCTTGTATGTTCCGGAAGACGGGTTGGGGCGCGCCGCACTTCGACCTCCTGGGGCTTAAATTCGGTTC		
		AM1G13	
	3' OH 5' P		AM1G12
CAACATTCATGCACAGCATCAACGGCTAC	GTGTTGCACAGCGCTGCAGCTGAGCGGTGCTTCCTGCACAGAGTGGCCTACTGGTACATCCTGAG		
GTGTGA	GTACGTGTCTGAGTTGCCGATG	CAACAGCTGTTCGAGCGTCCGACTCCGACACAGGATGTGTTCCACCGGATGACATGTAGGACTTC	
5' P 3' OH			AM1G12
	3' OH 5' P		
CATCGGGCCCCAGACCGACTTCCTGAGCGGTTCCTTCAGC	TGGCTACACCTTCACAGCACAAGATG	GTGTACGAGGACACCGCTGACCGTGT	
GTAGCCCGCGGCTCTGGCTGAAGGACTCGCACAAAGAAATCG	CCGATGTGAAGTTCCGTGTTCAC	CACATGCTCCTGTGGACTGGACAA	
	5' P 3' OH		
		AM1G13	
		BamHI	HindIII
CCCCCTTCAGCGGTGAGACCGGTTCATGAGCAATGAGAACCCCGCGCTGTGGATCCCTACAGAGCTTTAC			
GGGGAAGTCGCCCTCTGTGGCAAAATACCTTCCTTGGGGCCGGAACCTAAGGATGTTCGAATG			
		AM1G11	

FIG. 5 (7 of 14)

## Fragment H

[illegible]

FIG. 5 (8 of 14)

# Fragment I

[illegible]GCCTTAC  
CTTATG

**FIG. 5 (9 of 14)**

1. *Chlorophyll a* (Chl *a*) content was determined using a spectrophotometer (Shimadzu UV-160U) at 663 nm. The concentration of Chl *a* was calculated using the following equation:  $\text{Chl } a \text{ (mg/L)} = 12.7 \times \text{OD}_{663}$ .

2. *Chlorophyll b* (Chl *b*) content was determined using a spectrophotometer (Shimadzu UV-160U) at 646 nm. The concentration of Chl *b* was calculated using the following equation:  $\text{Chl } b \text{ (mg/L)} = 22.9 \times \text{OD}_{646}$ .

3. *Carotenoid* content was determined using a spectrophotometer (Shimadzu UV-160U) at 440 nm. The concentration of carotenoids was calculated using the following equation:  $\text{Carotenoid (mg/L)} = 24.4 \times \text{OD}_{440}$ .

4. *Protein* content was determined using a protein assay kit (Bio-Rad) according to the manufacturer's instructions. The concentration of protein was calculated using the following equation:  $\text{Protein (mg/L)} = 1.55 \times \text{OD}_{595}$ .

5. *Glucose* content was determined using a glucose assay kit (Bio-Rad) according to the manufacturer's instructions. The concentration of glucose was calculated using the following equation:  $\text{Glucose (mg/L)} = 1.55 \times \text{OD}_{595}$ .

6. *Starch* content was determined using a starch assay kit (Bio-Rad) according to the manufacturer's instructions. The concentration of starch was calculated using the following equation:  $\text{Starch (mg/L)} = 1.55 \times \text{OD}_{595}$ .

7. *Cellulose* content was determined using a cellulose assay kit (Bio-Rad) according to the manufacturer's instructions. The concentration of cellulose was calculated using the following equation:  $\text{Cellulose (mg/L)} = 1.55 \times \text{OD}_{595}$ .

8. *Lignin* content was determined using a lignin assay kit (Bio-Rad) according to the manufacturer's instructions. The concentration of lignin was calculated using the following equation:  $\text{Lignin (mg/L)} = 1.55 \times \text{OD}_{595}$ .

9. *Cellulose* content was determined using a cellulose assay kit (Bio-Rad) according to the manufacturer's instructions. The concentration of cellulose was calculated using the following equation:  $\text{Cellulose (mg/L)} = 1.55 \times \text{OD}_{595}$ .

10. *Lignin* content was determined using a lignin assay kit (Bio-Rad) according to the manufacturer's instructions. The concentration of lignin was calculated using the following equation:  $\text{Lignin (mg/L)} = 1.55 \times \text{OD}_{595}$ .

**EcoRI** **BstEII** **AMI Jf1**  
 GTAGAATTCGTAGGGTGAACCTTCCGCAACCAAGGCCAGCCCCCTACAGCTTCTACAGACAGCCCTGATCAGCTACGAGGAGCAGCGCC  
 CATCTTAAAGCATCCCACTGGAAGGCTTCTCTCCGGTCCGGCGGGGATGTGGAAGATGTCTGGACTAGTCGATGCTCTCTCTGTCGGCG

3' OH 5' P  
**AMI Jf3** **EagI** **BstEII** **HindIII**  
 GTGTGCAACCAACCAACACCTGAACCCCGCCAGCGGCGGCGGAGTGACCTTACAAAGCTTTTAC  
 CACACGGTGTGTGTGTGGACTTGGGGCGGTTGCCGGCGGTCCACTGGGATGTTGAAATTC

**AMI Jf1**

3' OH 5' P  
**AMI Jf2**  
 AGG\_TGCGCCGAGCCCCCGCAAGAACTTC GTGAAGCCCCCAAGAGACCAAGACCTACTTCTGGAAAGGTGCAAGCACCACATGGCCCCCACCAC  
 TCC CGCGGCTCGGGGCGCTTCTTGAAAGCTACCTTCGGGGTTGCTCTGGTCTCGATGAAATGATCTTCCACGTCGTGGTGTACCGGGGGTGGTTT

3' OH 5' P  
**AMI Jf2**  
 GGACGAGTTCGACTGCACAGGCGCTGGGCGCTTACCTTCAGCGACCTGGAGAGAGC GTGCACAGCGGCGCTGATCGCCCCCTGCTTC  
 CCTCTCAGACTGACGTTCCGGACCCCGGATGAAATTC GTTCGACCTGGAACCTTCTCTTCGCTACGTTGCCGAGCTAGCCGGGGAGAGAC

3' OH 5' P  
**AMI Jf3** **EagI** **BstEII** **HindIII**  
 GTGTGCAACCAACCAACACCTGAACCCCGCCAGCGGCGGCGGAGTGACCTTACAAAGCTTTTAC  
 CACACGGTGTGTGTGTGGACTTGGGGCGGTTGCCGGCGGTCCACTGGGATGTTGAAATTC

**AMI Jf1**

Variable	Mean	SD	Min	Max	Skewness	Kurtosis	Normality
Age	35.2	12.5	18	65	0.15	3.2	0.98
Gender	0.52	0.50	0	1	0.02	3.0	0.99
Marital Status	0.65	0.48	0	1	0.05	3.1	0.99
Education	12.8	2.1	9	16	0.10	3.3	0.98
Income	4500	1500	1000	10000	0.20	3.5	0.97
Occupation	0.45	0.50	0	1	0.03	3.0	0.99
Health Status	0.75	0.43	0	1	0.08	3.2	0.98
Stress Level	3.2	1.5	1	5	0.12	3.4	0.98
Life Satisfaction	4.1	1.2	1	5	0.09	3.3	0.98
Resilience	3.8	1.1	1	5	0.11	3.4	0.98
Optimism	4.3	1.0	1	5	0.07	3.2	0.99
Emotional Stability	3.9	1.3	1	5	0.13	3.5	0.97
Self-Esteem	4.0	1.1	1	5	0.08	3.3	0.98
Life Satisfaction	4.1	1.2	1	5	0.09	3.3	0.98
Resilience	3.8	1.1	1	5	0.11	3.4	0.98
Optimism	4.3	1.0	1	5	0.07	3.2	0.99
Emotional Stability	3.9	1.3	1	5	0.13	3.5	0.97
Self-Esteem	4.0	1.1	1	5	0.08	3.3	0.98

EcoRI	KpnI	AM1K1	PmlI
GATGAATTCGATGGGTACCTGCTGAGCATGCGCAGCAAGACAATCCACAGCATCCACTTCAGCGGCCACCGTGTTCACCGTGGCAAGAA			
CATCTTTAAGCATCCCATGGACGACTGCTGATGCTGCTGCTTGTCTTGTAGGTGTGATGTTGAAAGTTCGCCGTGCACAAAGTTTCTATCTGGTTCTTT		AM1K1	
		AM1K13	
	3' OH 5' P		AM1K12
GGAGTGATTCACAAGATGGCCCTGTACAACTGTACACCCCGCGCTGTTCGAGACCGTGAGATGCTGCCAGCAAGCCCGGCATCTGGCGGT			
CTCTCTGTTCATGTTCTTACCGGAGACATGTTCTATACATGGGGCCGACAAAGCTCTGGCACTCTTCTACGGGTGTTCTGGCTTAGACTCTCTTA	5' P 3' OH		AM1K2
		3' OH 5' P	
GGAGTGCCTGATCGCGCAGACACCTGCACGCCCGCATGAGTACCCCTGTCTCTGTGTACAGCAACCAAGTCCAGACCCCCCTGGGCATGGC			
CTCTACGGACTAGCCGCTCTGTGAGCGTGCACCGCTACTGTGTGGGACAAGAACCATATGTCGTTGTTCACGGTCTCTGGGGGACCCGTTACCG	5' P 3' OH		
	AM1K13	Apal	HindIII
CATACCGGCACATCTCTGCACTTCAGATTCACCCACAGCGGCCAGTACGGCCAGTGGGCCCTTACAAAGCTTTTAC			
CTCTCTCGGTTGAAAGAGCTGAAAGTCTATATCTCTTCGCCGCTCATGCCGTTACCCGGTCAACCCGCTATATCTTTCGAAATATG			
AM1K1			

[illegible]

# Fragment L

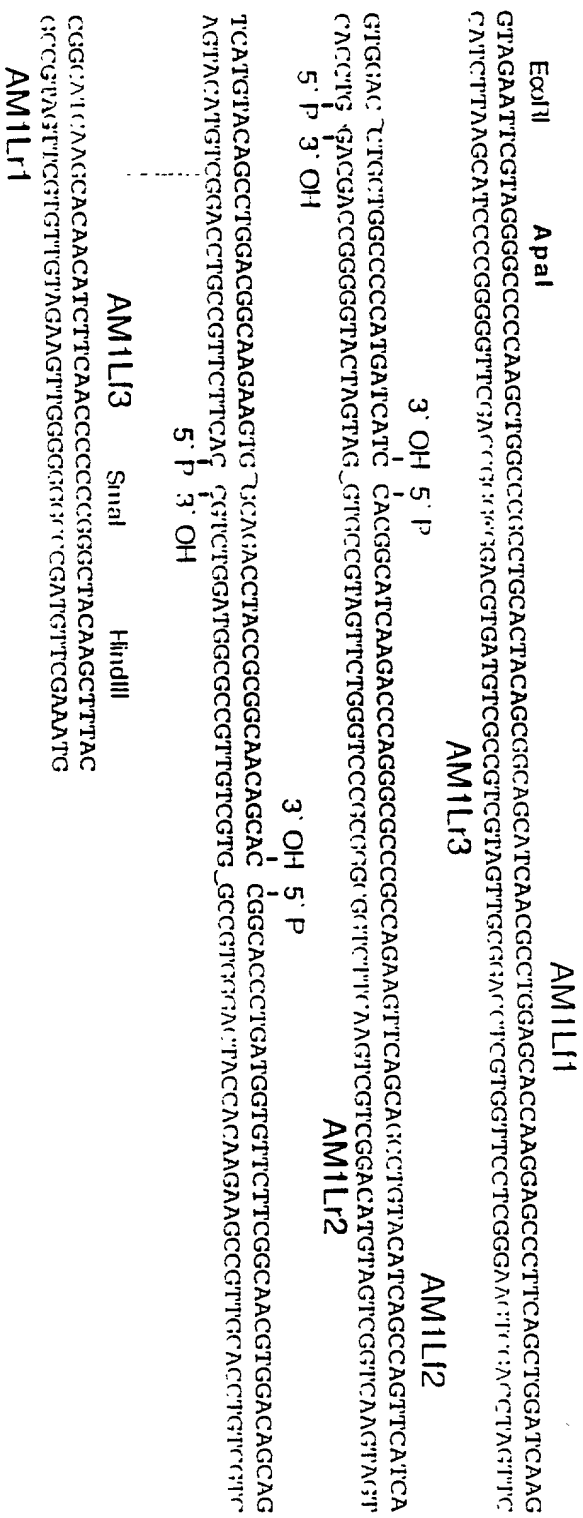


FIG. 5 (12 of 14)



# Fragment M

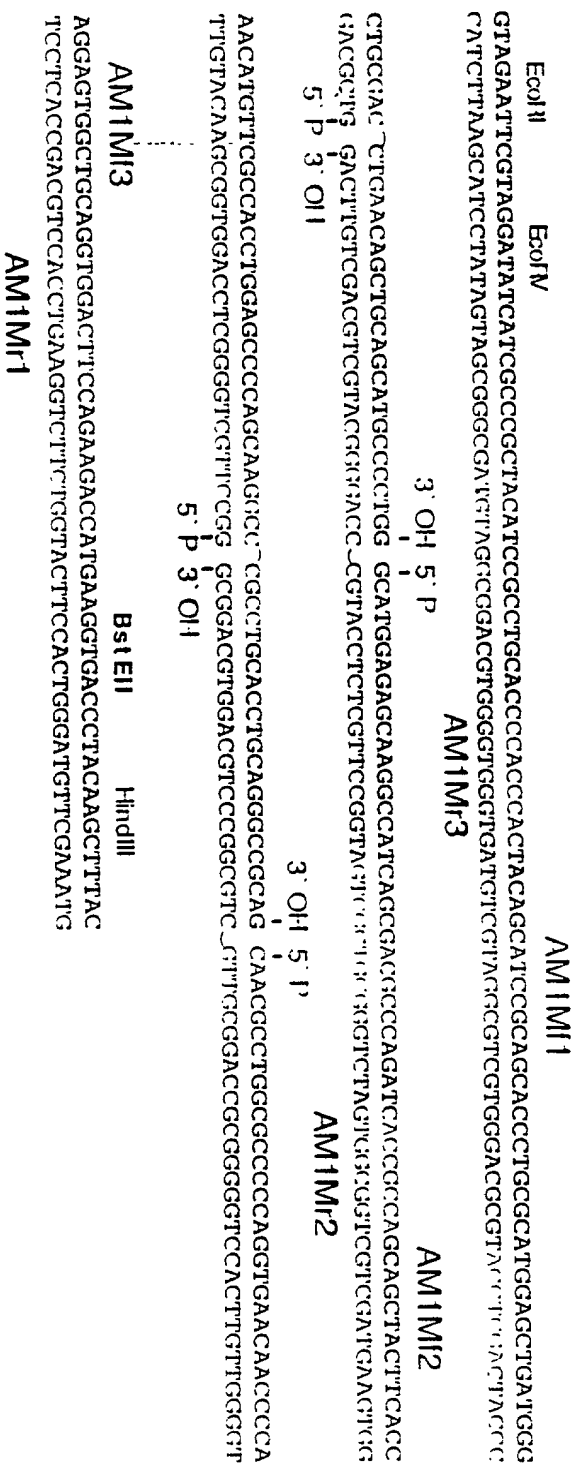


FIG. 5 (13 of 14)

## Fragment N

[illegible]

**FIG. 5 (14 of 14)**

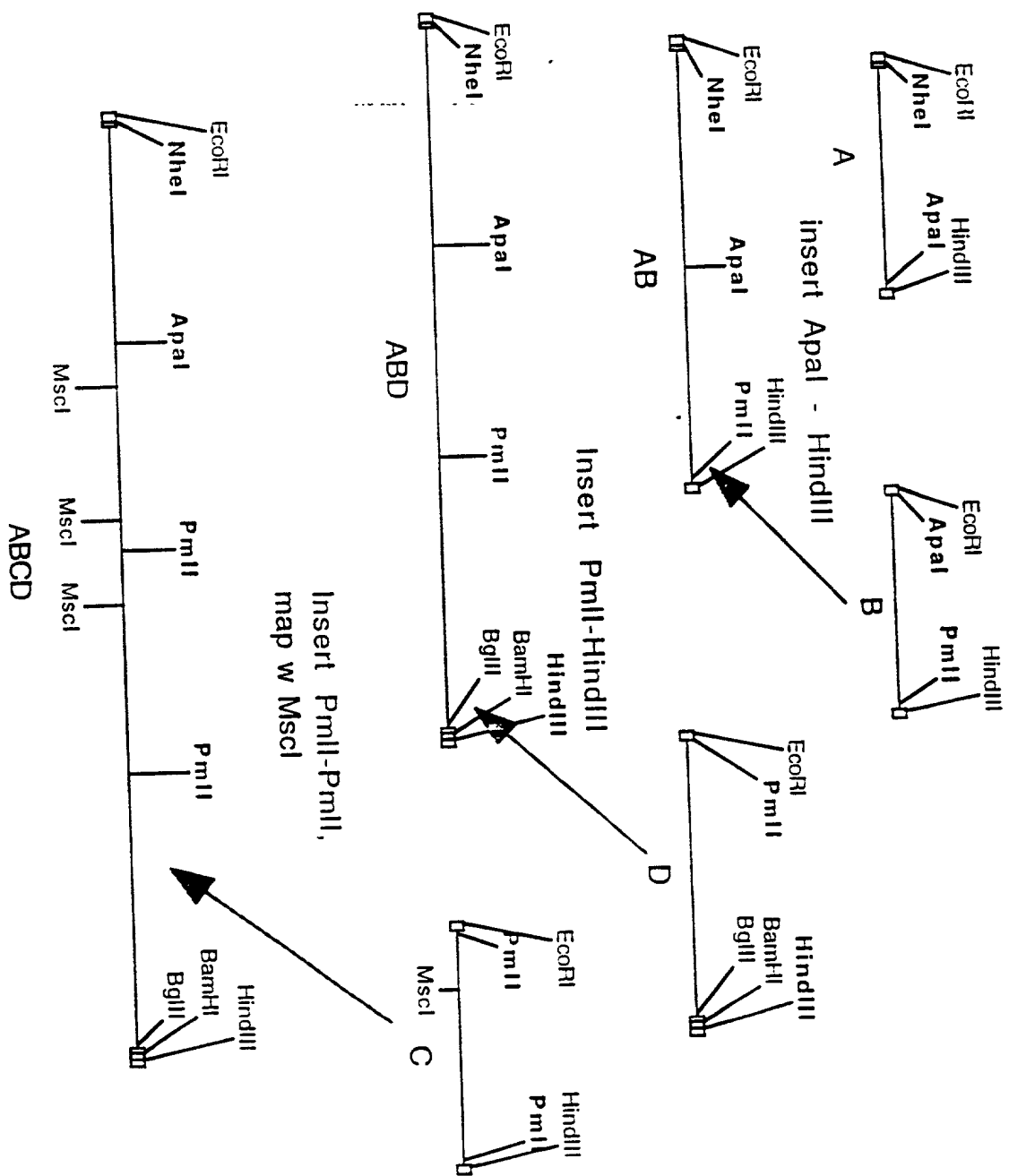


FIG. 6 (1 of 5)

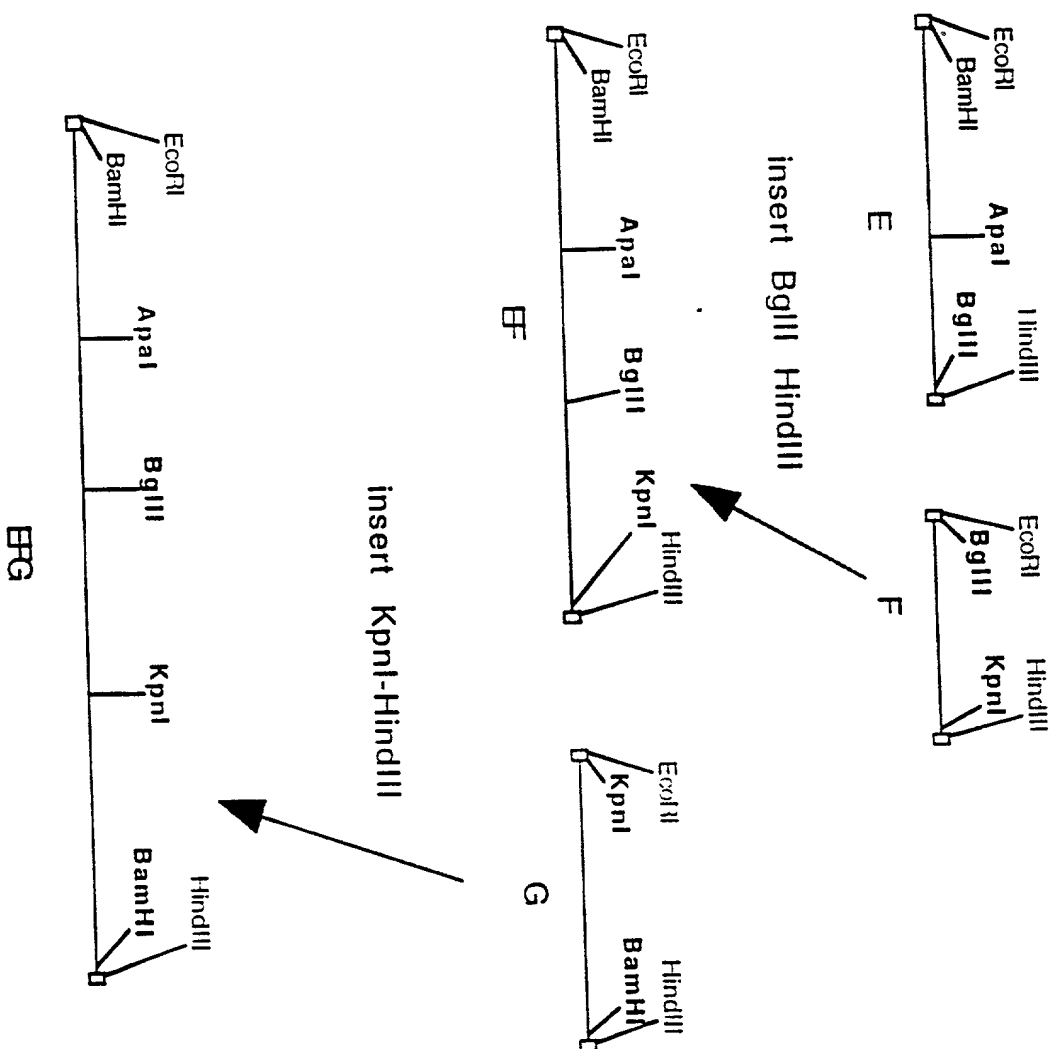


FIG. 6 (2 of 5)

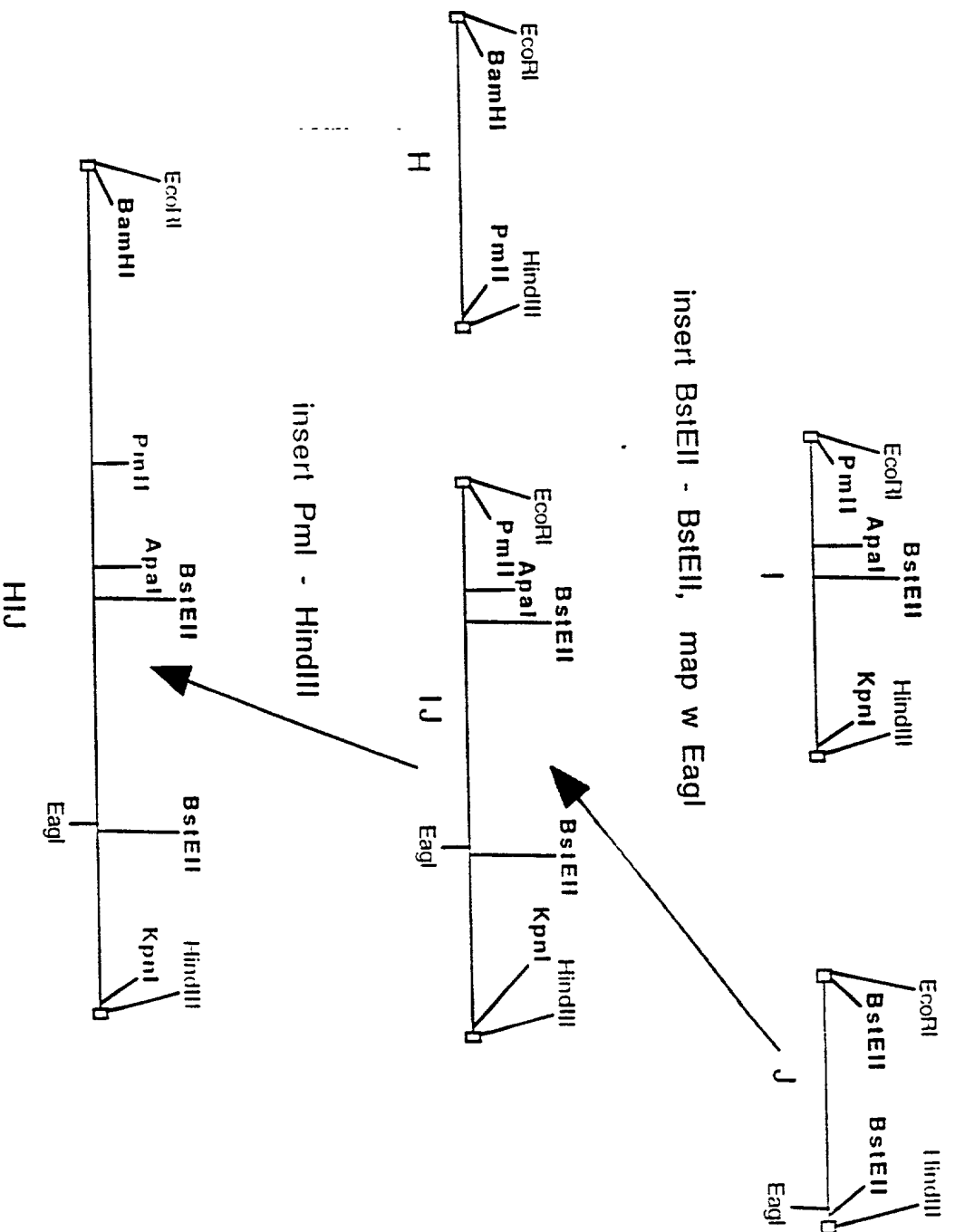


FIG. 6 (3 of 5)

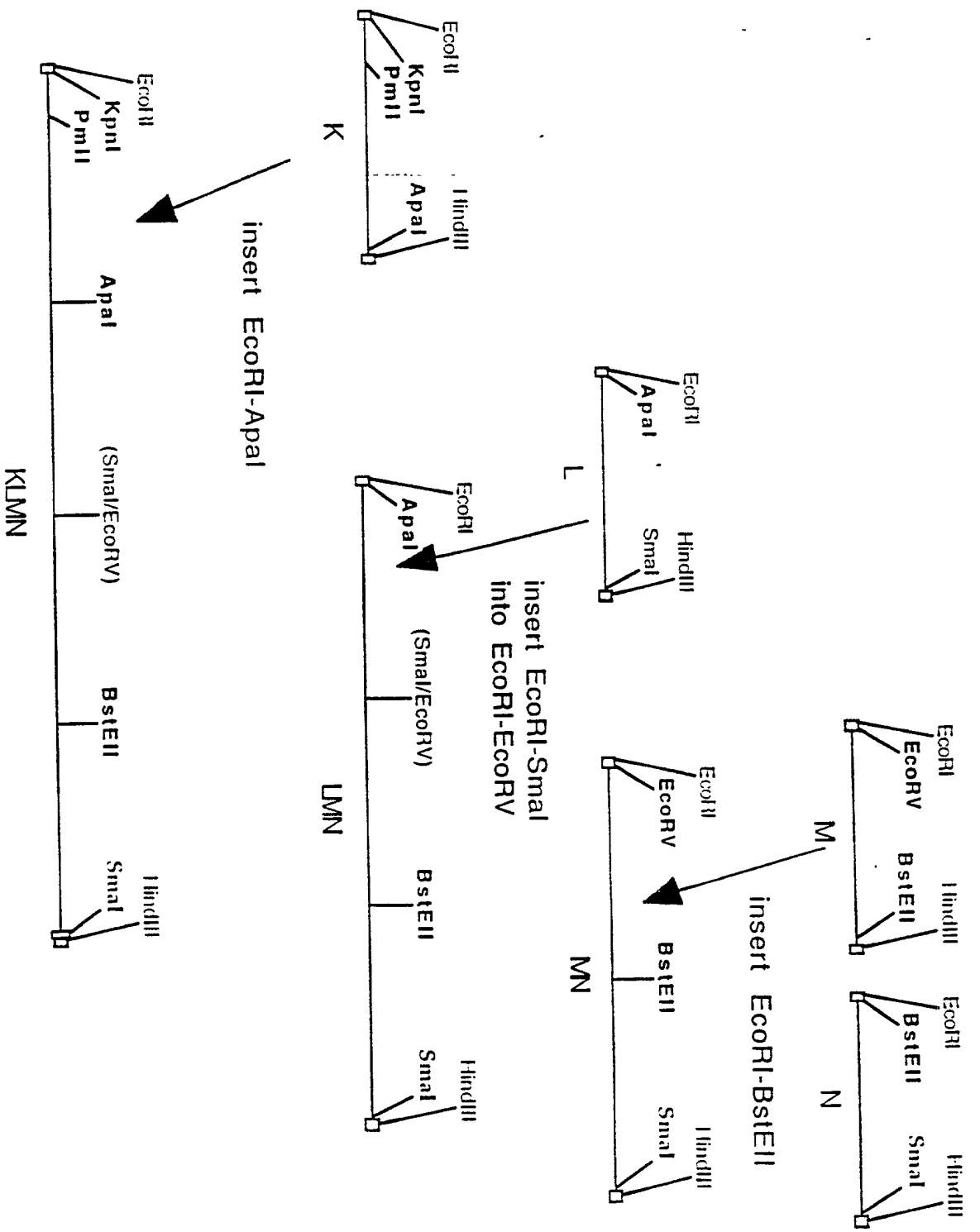


FIG. 6 (4 of 5)

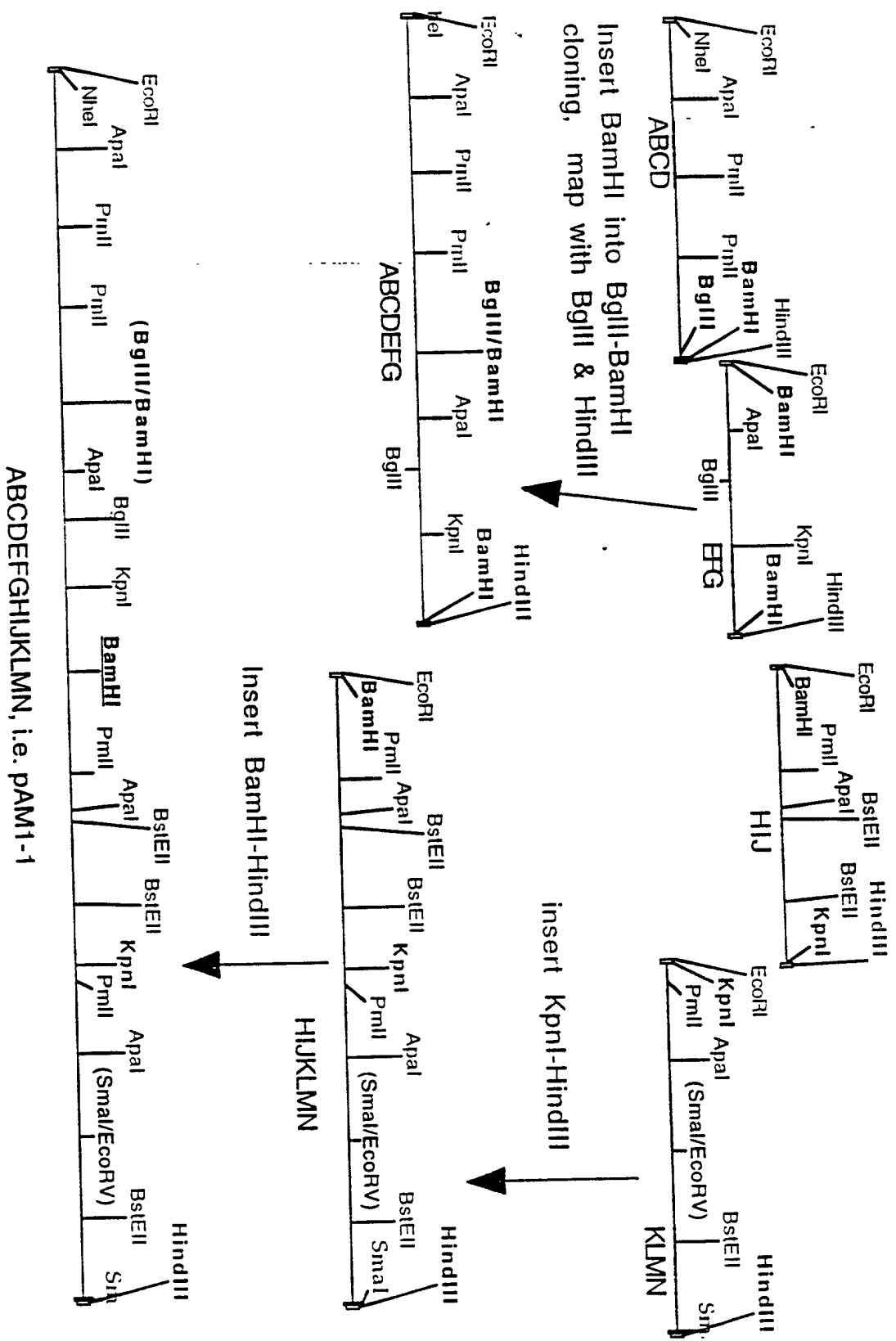


FIG. 6 (5 of 5)

EcoRI NheI  
 1 TAGAATTCGTAGGCTAGCATGCAGATCGAGCTGAGCACCTGCTTCTTCTCTGTGCCTGCTGCGCTTCTGCTTC  
 1 MetGlnIleGluLeuSerThrCysPhePheLeuCysLeuLeuArgPheCysPhe  
 73 AGCGCCACCCGCGCTACTACCTGGGCGCGCTGGAGCTGAGCTGGGACTACATGCAGAGCGACCTGGGCGAG  
 19 SerAlaThrArgArgTyrTyrLeuGlyAlaValGluLeuSerTrpAspTyrMetGlnSerAspLeuGlyGlu  
 145 CTGCCCCGTGGACGCCCCGCTTCCCCCCCCCGCTGCCAAGAGCTTCCCCCTCAACACCAGCGTGGTGTACAAG  
 43 LeuProValAspAlaArgPheProProArgValProLysSerPheProPheAsnThrSerValValTyrLys  
 217 AAGACCCTGTTCGTGGAGTTCACCGACCACCTGTTCAACATCGCCAAAGCCCCGCCCCCTGGATGGGCGCTG  
 67 LysThrLeuPheValGluPheThrAspHisLeuPheAsnIleAlaLysProArgProProTrpMetGlyLeu  
 Apal MscI  
 289 CTGGGCCCCACCATCCAGGCCGAGGTGTACGACACCGTGGTGATCACCTGAAGAACATGGCCAGCCACCCC  
 91 LeuGlyProThrIleGlnAlaGluValTyrAspThrValValIleThrLeuLysAsnMetAlaSerHisPro  
 361 GTGAGCCTGCACGCGCTGGGCGTGAGCTACTGGAAGGCCAGCGAGGGCGCCGAGTACGACGACCAGACCAGC  
 115 ValSerLeuHisAlaValGlyValSerTyrTrpLysAlaSerGluGlyAlaGluTyrAspAspGlnThrSer  
 433 CAGCGCGAGAAGGAGGACGACAAGGTGTTCCCCGGCGGCAGCCACACCTACGTGTGGCAGGTGCTGAAGGAG  
 139 GlnArgGluLysGluAspAspLysValPheProGlyGlySerHisThrTyrValTrpGlnValLeuLysGlu  
 MscI PmlI  
 505 AACGGCCCCATGGCCAGCGACCCCCCTGTGCCTGACCTACAGCTACCTGAGCCACGTGGACCTGGTGAAGGAC  
 163 AsnGlyProMetAlaSerAspProLeuCysLeuThrTyrSerTyrLeuSerHisValAspLeuValLysAsp  
 MscI  
 577 CTGAACAGCGGCCTGATCGGCGCCCTGCTGGTGTGCCGCGAGGCGAGCCTGGCCAAGGAGAAGACCCAGACC  
 187 LeuAsnSerGlyLeuIleGlyAlaLeuLeuValCysArgGluGlySerLeuAlaLysGluLysThrGlnThr  
 649 CTGCACAAGTTCATCCTGCTGTTCCGCGTGTTCGACGAGGGCAAGAGCTGGCACAGCGAGACCAAGAACAGC  
 211 LeuHisLysPheIleLeuLeuPheAlaValPheAspGluGlyLysSerTrpHisSerGluThrLysAsnSer  
 721 CTGATGCAGGACCGCGACGCGCCAGCGCCCGCGCTGGCCCAAGATGCACACCGTGAACGGCTACGTGAAC  
 235 LeuMetGlnAspArgAspAlaAlaSerAlaArgAlaTrpProLysMetHisThrValAsnGlyTyrValAsn  
 PmlI  
 793 CGCAGCCTGCCCCGGCCTGATCGGCTGCCACCGCAAGAGCGTGTACTGGCACGTGATCGGCATGGGCACCACC  
 259 ArgSerLeuProGlyLeuIleGlyCysHisArgLysSerValTyrTrpHisValIleGlyMetGlyThrThr  
 865 CCCGAGGTGCACAGCATCTTCTCGGAGGGCCACACCTTCTCGGTGCGCAACCACCGCCAGGCCAGCCTGGAG  
 283 ProGluValHisSerIlePheLeuGluGlyHisThrPheLeuValArgAsnHisArgGlnAlaSerLeuGlu  
 937 ATCAGCCCCATCACCTTCTGACCGCCCGAGACCCTGCTGATGGACCTGGGCCAGTTCCTGCTGTCTGCCAC  
 307 IleSerProIleThrPheLeuThrAlaGlnThrLeuLeuMetAspLeuGlyGlnPheLeuLeuPheCysHis  
 1009 ATCAGCAGCCACCAGCAGCAGCGCATGGAGGCCTACGTGAAGGTGGACAGCTGCCCCGAGGAGCCCCAGCTG  
 331 IleSerSerHisGlnHisAspGlyMetGluAlaTyrValLysValAspSerCysProGluGluProGlnLeu  
 1081 CGCATGAAGAACAACGAGGAGGCCGAGGACTACGACGACGACCTGACCGACAGCGAGATGGACGTGGTGGCGC  
 355 ArgMetLysAsnAsnGluGluAlaGluAspTyrAspAspAspLeuThrAspSerGluMetAspValValArg  
 (BglII/BamHI)  
 1153 TTCGACGACGACAACAGCCCCAGCTTCATCCAGATCCGCAGCGTGGCCAAGAAGCACCCCAAGACCTGGGTG  
 379 PheAspAspAspAsnSerProSerPheIleGlnIleArgSerValAlaLysLysHisProLysThrTrpVal  
 1225 CACTACATCGCCGCGGAGGAGGAGGACTGGGACTACGCCCCCTGGTGTGGCCCCGACGACCGCAGCTAC  
 403 HisTyrIleAlaAlaGluGluGluAspTrpAspTyrAlaProLeuValLeuAlaProAspAspArgSerTyr  
 EagI  
 1297 AAGAGCCAGTACCTGAACAACGGCCCCCAGCGCATCGGCGCGCAAGTACAAGAAGGTGCGCTTCATGGCCTAC  
 427 LysSerGlnTyrLeuAsnAsnGlyProGlnArgIleGlyArgLysTyrLysLysValArgPheMetAlaTyr  
 Apal  
 1369 ACCGACGAGACCTTCAAGACCCGCGAGGCCATCCAGCAGGAGAGCGGCATCCTGGGCCCCCTGCTGTACGGC  
 451 ThrAspGluThrPheLysThrArgGluAlaIleGlnHisGluSerGlyIleLeuGlyProLeuLeuTyrGly

FIG. 7 (1 of 3)



1441 CAGGTGGGCGACACCCTGCTGATCATCTTCAAGAACCAGGCCAGCCGCCCTACAACATCTACCCCCACGGC  
 475▶ GluValGlyAspThrLeuLeuIleIlePheLysAsnGlnAlaSerArgProTyrAsnIleTyrProHisGly  
 1513 ATCACCACGTGCGCCCCCTGTACAGCCGCCCTGCCCAAGGGCGTGAAGCACCTGAAGGACTTCCCCATC  
 499▶ IleThrAspValArgProLeuTyrSerArgArgLeuProLysGlyValLysHisLeuLysAspPheProIle

#### BglII

1585 CTGCCCCGGCGAGATCTTCAAGTACAAGTGGACCGTGACCGTGGAGGACGGCCCCACCAAGAGCGACCCCCGC  
 523▶ LeuProGlyGluIlePheLysTyrLysTrpThrValThrValGluAspGlyProThrLysSerAspProArg  
 1657 TGCCTGACCCGCTACTACAGCAGCTTCGTGAACATGGAGCGCGACCTGGCCAGCGGCCCTGATCGGCCCCCTG  
 547▶ CysLeuThrArgTyrTyrSerSerPheValAsnMetGluArgAspLeuAlaSerGlyLeuIleGlyProLeu  
 1729 CTGATCTGCTACAAGGAGAGCGTGGACCAGCGCGCAACCAGATCATGAGCGACAAGCGCAACGTGATCCTG  
 571▶ LeuIleCysTyrLysGluSerValAspGlnArgGlyAsnGlnIleMetSerAspLysArgAsnValIleLeu

#### KpnI

1801 TTCAGCGTGTTCGACGAGAACCGCAGCTGGTACCTGACCGAGAACATCCAGCGCTTCCTGCCCCAACCCCGCC  
 595▶ PheSerValPheAspGluAsnArgSerTrpTyrLeuThrGluAsnIleGlnArgPheLeuProAsnProAla  
 1873 GCGCTGCAGCTGGAGGACCCCGAGTTCCAGGCCAGCAACATCATGCACAGCATCAACGGCTACGTGTTTCGAC  
 619▶ GlyValGlnLeuGluAspProGluPheGlnAlaSerAsnIleMetHisSerIleAsnGlyTyrValPheAsp  
 1945 AGCCTGCAGCTGAGCGTGTGCCTGCACGAGGTGGCCTACTGGTACATCCTGAGCATCGGGCGCCAGACCGAC  
 643▶ SerLeuGlnLeuSerValCysLeuHisGluValAlaTyrTrpTyrIleLeuSerIleGlyAlaGlnThrAsp  
 2017 TTCCTGAGCGTGTCTTTCAGCGGCTACACCTTCAAGCACAAGATGGTGTACGAGGACACCCTGACCCTGTTC  
 667▶ PheLeuSerValPhePheSerGlyTyrThrPheLysHisLysMetValTyrGluAspThrLeuThrLeuPhe

#### BamHI

2089 CCCTTCAGCGGCGAGACCGTGTTCATGAGCATGGAGAACCCCGGCCTGTGGATCCTGGGCTGCCACAACAGC  
 691▶ ProPheSerGlyGluThrValPheMetSerMetGluAsnProGlyLeuTrpIleLeuGlyCysHisAsnSer  
 2161 GACTTCCGCAACCGCGGCATGACCGCCCTGCTGAAGGTGAGCAGCTGCGACAAGAACACCGGCGACTACTAC  
 715▶ AspPheArgAsnArgGlyMetThrAlaLeuLeuLysValSerSerCysAspLysAsnThrGlyAspTyrTyr  
 2233 GAGGACAGCTACGAGGACATCAGCGCCTACCTGCTGAGCAAGAACAACGCCATCGAGCCCCCGCTGGAGGAG  
 739▶ GluAspSerTyrGluAspIleSerAlaTyrLeuLeuSerLysAsnAsnAlaIleGluProArgLeuGluGlu

#### BstXI

2305 ATCACC CGCACCACCTGCAGAGCGACCAGGAGGAGATCGACTACGACGACACCATCAGCGTGGAGATGAAG  
 763▶ IleThrArgThrThrLeuGlnSerAspGlnGluGluIleAspTyrAspAspThrIleSerValGluMetLys  
 2377 AAGGAGGACTTCGACATCTACGACGAGGACGAGAACCAGAGCCCCCGCAGCTTCCAGAAGAAGACCCGCCAC  
 787▶ LysGluAspPheAspIleTyrAspGluAspGluAsnGlnSerProArgSerPheGlnLysLysThrArgHis

#### PmlI

2449 TACTTCATCGCCCGCGTGGAGCGCCTGTGGGACTACGGCATGAGCAGCAGCCCCACGTGCTGCGCAACCGC  
 811▶ TyrPheIleAlaAlaValGluArgLeuTrpAspTyrGlyMetSerSerSerProHisValLeuArgAsnArg  
 2521 GCCCAGAGCGGCAGCGTGCCCCAGTTCAAGAAGGTGGTGTTCAGGAGTTCACCGACGGCAGCTTCACCCAG  
 835▶ AlaGlnSerGlySerValProGlnPheLysLysValValPheGlnGluPheThrAspGlySerPheThrGln

#### Apal

2593 CCCCTGTACCGCGGCGAGCTGAACGAGCACCTGGGCCTGCTGGGCCCTACATCCGCGCCGAGGTGGAGGAC  
 859▶ ProLeuTyrArgGlyGluLeuAsnGluHisLeuGlyLeuLeuGlyProTyrIleArgAlaGluValGluAsp

#### BstEII

2665 AACATCATGGTGAACCTTCGCAACCAGGCCAGCCGCCCTACAGCTTCTACAGCAGCCTGATCAGCTACGAG  
 883▶ AsnIleMetValThrPheArgAsnGlnAlaSerArgProTyrSerPheTyrSerSerLeuIleSerTyrGlu  
 2737 GAGGACGAGCGCCAGGGCGCCGAGCCCCGCAAGAAGTTCGTGAAGCCCAACGAGACCAAGACCTACTTCTGG  
 907▶ GluAspGlnArgGlnGlyAlaGluProArgLysAsnPheValLysProAsnGluThrLysThrTyrPheTrp  
 2809 AAGGTGCAGCACCATGGCCCCACCAAGGACGAGTTCGACTGCAAGGCCTGGGCCTACTTCAGCGACGTG  
 931▶ LysValGlnHisHisMetAlaProThrLysAspGluPheAspCysLysAlaTrpAlaTyrPheSerAspVal

FIG. 7 (2 of 3)



EcoRI BamHI  
 GTAGATTCGGATCCCTGGCTGCCACACAGCGACTTCCGCAACCGCGGCATTCACCCGCTGTGATGATGAGCAGCTGCGACAAGACACCGGCGACTTACTAC  
 CATCTTAAAGCCTAGGACCCGACGGGTGTGTGCTGTAAATACGTTGGCGCCGTACTGTGGGACGAACTTCTTCGTCGACGCTGTCTTGTGGATGGATGATGATG  
 AM8R4  
 AM8FR2  
 3' OH 5' P  
 5' P 3' OH  
 GAGGACAGCTACGAGTACATCAGCGCCTACCTGCTGAGCAAGAACACAGCCCATCGAGCCCCCGCAGGCGCAGGTCGAGATCACCCGACACACCCTGCAGA  
 CTTCCTGTTCGATGCTCTGTGATGTCGCGGATGGACGACCTTCGTTCTTGTGTGCGGTAGCTTCGGGGCGTCCGCGTCCGCGTCCGCTCTAGTGGCGGTGGTGGAGCGTCTP  
 5' P 3' OH  
 AM8BR2  
 5' P 3' OH  
 AM8F4 BstXI HindIII  
 GCCAATCAGGAGAGATCGACTACGACGACACCATCAGCGTGAAGCTTTAC  
 CGCTGCTCCTCCTTAGCTGATGCTGCTGTGTAGTTCGACCTTCGAATG  
 AM8R1

FIG. 8

EcoRI NheI

1 TAGAATTCCTAGGCTAGCATGCAGATCGAGCTGAGCACCTGCTTCTTCTCTGTCCTGCTGCGCTTCTGCTTC  
1 MetGlnIleGluLeuSerThrCysPhePheLeuCysLeuLeuArgPheCysPhe

73 AGCGCCACCCGCGCTACTACCTGGGCGCCCTGGAGCTGAGCTGGGACTACATGCAGAGCGACCTGGGCGAG  
19 SerAlaThrArgArgTyrTyrLeuGlyAlaValGluLeuSerTrpAspTyrMetGlnSerAspLeuGlyGlu

145 CTGCCCCGTGGACGCCCCGCTTCCCCCCCCCGCGTGGCCCAAGAGCTTCCCCCTCAACACCAGCGTGGTGTACAAG  
43 LeuProValAspAlaArgPheProProArgValProLysSerPheProPheAsnThrSerValValTyrLys

217 AAGACCCTGTTCGTGGAGTTCACCGACCACCTGTTCACACATCGCCAAGCCCCGCCCCCCCCCTGGATGGGCCTG  
67 LysThrLeuPheValGluPheThrAspHisLeuPheAsnIleAlaLysProArgProProTrpMetGlyLeu

Apal MscI

289 CTGGGCCCCACCATCCAGGCCGAGGTGTACGACACCGTGGTGATCACCTGAAGAACATGGCCAGCCACCCC  
91 LeuGlyProThrIleGlnAlaGluValTyrAspThrValValIleThrLeuLysAsnMetAlaSerHisPro

361 CTGAGCCTGCACGCGCTGGGCGTGAGCTACTGGAAGGCCAGCGAGGGCGCCGAGTACGACGACCAGACCAGC  
115 ValSerLeuHisAlaValGlyValSerTyrTrpLysAlaSerGluGlyAlaGluTyrAspAspGlnThrSer

433 CAGCGCGAGAAGGAGGACGACAAGGTGTTCCCCGGCGGCAGCCACACCTACGTGTGGCAGGTGCTGAAGGAG  
139 GlnArgGluLysGluAspAspLysValPheProGlyGlySerHisThrTyrValTrpGlnValLeuLysGlu

MscI PmlI

505 AACGGCCCCATGGCCAGCGACCCCCCTGTGCCTGACCTACAGCTACCTGAGCCACGTGGACCTCGTGAAGGAC  
163 AsnGlyProMetAlaSerAspProLeuCysLeuThrTyrSerTyrLeuSerHisValAspLeuValLysAsp

MscI

577 CTGAACAGCGGCCTGATCGGCGCCCTGCTGGTGTGCCGCGAGGGCAGCCTGGCCAAGGAGAAGACCCAGACC  
187 LeuAsnSerGlyLeuIleGlyAlaLeuLeuValCysArgGluGlySerLeuAlaLysGluLysThrGlnThr

649 CTGCACAAGTTCATCCTGCTGTTCGCCGTGTTTCGACGAGGGCAAGAGCTGGCACAGCGAGACCAAGAACAGC  
211 LeuHisLysPheIleLeuLeuPheAlaValPheAspGluGlyLysSerTrpHisSerGluThrLysAsnSer

721 CTGATGCAGGACCGCGACGCGCCGACGCCCCGCGCTGGCCCCAAGATGCACACCGTGAACGGCTACGTGAAC  
235 LeuMetGlnAspArgAspAlaAlaSerAlaArgAlaTrpProLysMetHisThrValAsnGlyTyrValAsn

PmlI

793 CGCAGCCTGCCCCGCCTGATCGGCTGCCACCGCAAGAGCGTGTACTGGCACGTGATCGGCATGGGCACCACC  
259 ArgSerLeuProGlyLeuIleGlyCysHisArgLysSerValTyrTrpHisValIleGlyMetGlyThrThr

865 CCGGAGGTGCACAGCATCTTCTCGGAGGGCCACACCTTCTGGTGCGCAACCACCGCCAGGCCAGCCTGGAG  
283 ProGluValHisSerIlePheLeuGluGlyHisThrPheLeuValArgAsnHisArgGlnAlaSerLeuGlu

937 ATCAGCCCCATCACCTTCTGTGACCGCCGAGCCCTGCTGTGAGCCTGGGCCAGTTCCTGCTGTTCTGCCCAC  
307 IleSerProIleThrPheLeuThrAlaGlnThrLeuLeuMetAspLeuGlyGlnPheLeuLeuPheCysHis

1009 ATCAGCAGCCACCAGCACGACGGCATGGAGGCCTACGTGAAGGTGGACAGCTGCCCCGAGGAGCCCCAGCTG  
331 IleSerSerHisGlnHisAspGlyMetGluAlaTyrValLysValAspSerCysProGluGluProGlnLeu

1081 CGCATGAAGAACAACGAGGAGGCCGAGGACTACGACGACGACCTGACCGACAGCGAGATGGACGTGGTGGCG  
355 ArgMetLysAsnAsnGluGluAlaGluAspTyrAspAspAspLeuThrAspSerGluMetAspValValArg

(BglII/BamHI)

1153 TTCGACGACGACAACAGCCCCAGCTTCATCCAGATCCGACCGTGGCCAAGAAGCACCCCCAAGACCTGGGTG  
379 PheAspAspAspAsnSerProSerPheIleGlnIleArgSerValAlaLysLysHisProLysThrTrpVal

1225 CACTACATCGCCGCGGAGGAGGAGGACTGGGACTACGCCCCCTGGTGTGCTGGCCCCGACGACCGCAGCTAC  
403 HisTyrIleAlaAlaGluGluGluAspTrpAspTyrAlaProLeuValLeuAlaProAspAspArgSerTyr

EagI

1297 AAGAGCCAGTACCTGAACAACGGCCCCCAGCGCATCGGCCCAAGTACAAGAAGGTGCGCTTCATGGCCTAC  
427 LysSerGlnTyrLeuAsnAsnGlyProGlnArgIleGlyArgLysTyrLysLysValArgPheMetAlaTyr

Apal

1369 ACCGACGAGACCTTCAAGACCCGCGAGGCCATCCAGCACGAGAGCGGCATCCTGGGCCCCCTGCTGTACGGC  
451 ThrAspGluThrPheLysThrArgGluAlaIleGlnHisGluSerGlyIleLeuGlyProLeuLeuTyrGly

FIG. 9 (1 of 3)

1441 BAGGTGGGCGACACCCCTGCTGATCATCTTCAAGAACCAGGCCAGCCGCCCTACAACATCTACCCCCACGGC  
 475▶ GluValGlyAspThrLeuLeuIleIlePheLysAsnGlnAlaSerArgProTyrAsnIleTyrProHisGly  
 1513 ATCACCAGCGTGC GCCCCTGTACAGCCGCCCTGCCCCAAGGGCGTGAAGCACCTGAAGGACTTCCCCATC  
 499▶ IleThrAspValArgProLeuTyrSerArgArgLeuProLysGlyValLysHisLeuLysAspPheProIle

### BglII

1585 CTGCCCCGGCGAGATCTTCAAGTACAAGTGGACCGTGACCGTGGAGGACGGCCCCACCAAGAGCGACCCCCGC  
 523▶ LeuProGlyGluIlePheLysTyrLysTrpThrValThrValGluAspGlyProThrLysSerAspProArg  
 1657 TGCCTGACCCGCTACTACAGCAGCTTCGTGAACATGGAGCGCGACCTGGCCAGCGGCCTGATCGGCCCCCTG  
 547▶ CysLeuThrArgTyrTyrSerSerPheValAsnMetGluArgAspLeuAlaSerGlyLeuIleGlyProLeu  
 1729 CTGATCTGCTACAAGGAGAGCGTGGACCAGCGCGCAACCAGATCATGAGCGACAAGCGCAACGTGATCCTG  
 571▶ LeuIleCysTyrLysGluSerValAspGlnArgGlyAsnGlnIleMetSerAspLysArgAsnValIleLeu

### KpnI

1801 TTCAGCGTGTTCGACGAGAACCAGCTGGTACCTGACCGAGAACATCCAGCGCTTCCTGCCCCAACCCCGCC  
 595▶ PheSerValPheAspGluAsnArgSerTrpTyrLeuThrGluAsnIleGlnArgPheLeuProAsnProAla  
 1873 GGCGTGCAGCTGGAGGACCCGAGTTCCAGGCCAGCAACATCATGCACAGCATCAACGGCTACGTGTTTCGAC  
 619▶ GlyValGlnLeuGluAspProGluPheGlnAlaSerAsnIleMetHisSerIleAsnGlyTyrValPheAsp  
 1945 AGCCTGCAGCTGAGCGTGTGCCCTGCACGAGGTGGCCTACTGGTACATCCTGAGCATCGGCGCCCAGACCGAC  
 643▶ SerLeuGlnLeuSerValCysLeuHisGluValAlaTyrTrpTyrIleLeuSerIleGlyAlaGlnThrAsp  
 2017 TTCCTGAGCGTGTTCCTTCAGCGGTACACCTTCAAGCAACAAGATGGTGTACGAGGACACCCTGACCCTGTTC  
 667▶ PheLeuSerValPhePheSerGlyTyrThrPheLysHisLysMetValTyrGluAspThrLeuThrLeuPhe

### BamHI

2089 CCCTTCAGCGGCGAGACCGTGTTCATGAGCATGGAGAACCCCGGCCCTGTGGATCCTGGGCTGCCACAACAGC  
 691▶ ProPheSerGlyGluThrValPheMetSerMetGluAsnProGlyLeuTrpIleLeuGlyCysHisAsnSer  
 2161 GACTTCCGCAACCGCGGCATGACCGCCCTGCTGAAGGTGAGCAGCTGCGACAAGAACACCGGCGACTACTAC  
 715▶ AspPheArgAsnArgGlyMetThrAlaLeuLeuLysValSerSerCysAspLysAsnThrGlyAspTyrTyr  
 2233 GAGGACAGCTACGAGGACATCAGCGCCTACCTGCTGAGCAAGAACAACGCCATCGAGCCCCCGCAGGCGCAGG  
 739▶ GluAspSerTyrGluAspIleSerAlaTyrLeuLeuSerLysAsnAsnAlaIleGluProArgArgArgArg

### BstXI

2305 CGCGAGATCACCCGCACCACCCTGCAGAGCGACCAGGAGGAGATCGACTACGACGACACCATCAGCGTGGAG  
 763▶ ArgGluIleThrArgThrThrLeuGlnSerAspGlnGluGluIleAspTyrAspAspThrIleSerValGlu  
 2377 ATGAAGAAGGAGGACTTCGACATCTACGACGAGGACGAGAACCAGAGCCCCCGCAGCTTCCAGAAGAAGACC  
 787▶ MetLysLysGluAspPheAspIleTyrAspGluAspGluAsnGlnSerProArgSerPheGlnLysLysThr

### PmlI

2449 CGCCACTACTTCATCGCCGCCGTGGAGCGCCTGTGGGACTACGGCATGAGCAGCAGCCCCACGTGCTGCGC  
 811▶ ArgHisTyrPheIleAlaAlaValGluArgLeuTrpAspTyrGlyMetSerSerSerProHisValLeuArg  
 2521 AACC CGCCCCAGAGCGGCAGCGTGCCCCAGTTCAAGAAGGTGGTGTTCAGGAGTTCACCGACGGCAGCTTC  
 835▶ AsnArgAlaGlnSerGlySerValProGlnPheLysLysValValPheGlnGluPheThrAspGlySerPhe

### Apal

2593 ACCCAGCCCTGTACCGCGCGAGCTGAACGAGCACCTGGGCCTGCTGGGCCCCCTACATCCGCGCCGAGGTG  
 859▶ ThrGlnProLeuTyrArgGlyGluLeuAsnGluHisLeuGlyLeuLeuGlyProTyrIleArgAlaGluVal

### BstEII

2665 GAGGACAACATCATGGTGACCTTCCGCAACCAGGCCAGCCGCCCTACAGCTTCTACAGCAGCCTGATCAGC  
 383▶ GluAspAsnIleMetValThrPheArgAsnGlnAlaSerArgProTyrSerPheTyrSerSerLeuIleSer  
 2737 TACGAGGAGGACCAGCGCCAGGGCGCCGAGCCCCGCAAGAAGTTCGTGAAGCCCCACGAGACCAAGACCTAC  
 907▶ TyrGluGluAspGlnArgGlnGlyAlaGluProArgLysAsnPheValLysProAsnGluThrLysThrTyr  
 2809 TTCTGGAAGGTGCAGCACCATGGCCCCACCAAGGACGAGTTCGACTGCAAGGCCTGGGCCCTACTTCAGC  
 931▶ PheTrpLysValGlnHisHisMetAlaProThrLysAspGluPheAspCysLysAlaTrpAlaTyrPheSer

2881 BACGTGGACCTGGAGAAGGACGTGCACAGCGGCCTGATCGGCCCCCTGCTGGTGTGCCACACCAACACCCCTG  
 955▶ AspValAspLeuGluLysAspValHisSerGlyLeuIleGlyProLeuLeuValCysHisThrAsnThrLeu  
 EagI BstEII

2953 AACCCCGCCACCGCCCGCAGGTGACCGTGCAGGAGTTCGCCCTGTTCTTACCATCTTCGACGAGACCAAG  
 979▶ AsnProAlaHisGlyArgGlnValThrValGlnGluPheAlaLeuPhePheThrIlePheAspGluThrLys  
 3025 AGCTGGTACTTCACCGAGAACATGGAGCGCAACTGCCCGCGCCCCCTGCAACATCCAGATGGAGGACCCCACC  
 1003▶ SerTrpTyrPheThrGluAsnMetGluArgAsnCysArgAlaProCysAsnIleGlnMetGluAspProThr  
 3097 TTCAAGGAGAACTACCGCTTCCACGCCATCAACGGCTACATCATGGACACCCTGCCCGGCCTGGTGATGGCC  
 1027▶ PheLysGluAsnTyrArgPheHisAlaIleAsnGlyTyrIleMetAspThrLeuProGlyLeuValMetAla  
 KpnI

3169 CAGGACCAGCGCATCCGCTGGTACCTGCTGAGCATGGGCAGCAACGAGAACATCCACAGCATCCACTTCAGC  
 1051▶ GlnAspGlnArgIleArgTrpTyrLeuLeuSerMetGlySerAsnGluAsnIleHisSerIleHisPheSer  
 PmlI

3241 GGCCACGTGTTACCGTGCAGCAAGAAGGAGGAGTACAAGATGGCCCTGTACAACCTGTACCCCGGCGTGTTTC  
 1075▶ GlyHisValPheThrValArgLysLysGluGluTyrLysMetAlaLeuTyrAsnLeuTyrProGlyValPhe  
 3313 GAGACCGTGGAGATGCTGCCAGCAAGGCCGGCATCTGGCGCGTGGAGTGCCTGATCGGCGAGCACCTGCAC  
 1099▶ GluThrValGluMetLeuProSerLysAlaGlyIleTrpArgValGluCysLeuIleGlyGluHisLeuHis  
 3385 GCCGGCATGAGCACCCCTGTTCTCGGTGTACAGCAACAAGTGCCAGACCCCCCTGGGCATGGCCAGCGGCCAC  
 1123▶ AlaGlyMetSerThrLeuPheLeuValTyrSerAsnLysCysGlnThrProLeuGlyMetAlaSerGlyHis  
 ApaI

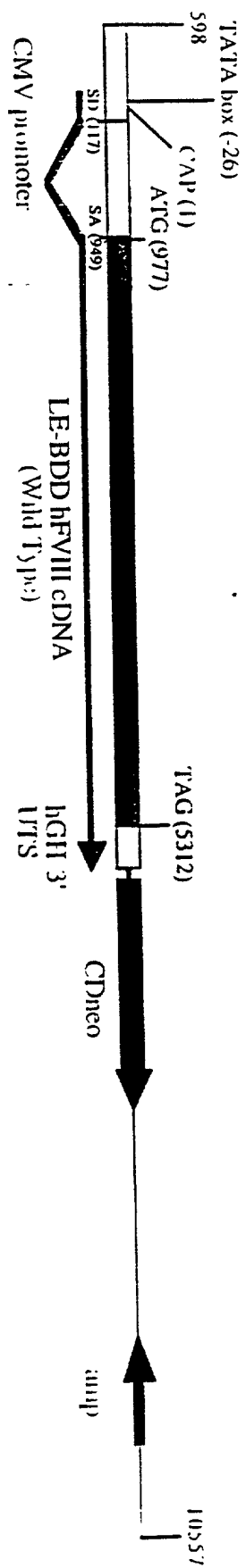
3457 ATCCGCGACTTCCAGATCACCGCCAGCGGCCAGTACGGCCAGTGGGCCCCCAAGCTGGCCCCGCTGCACTAC  
 1147▶ IleArgAspPheGlnIleThrAlaSerGlyGlnTyrGlyGlnTrpAlaProLysLeuAlaArgLeuHisTyr  
 3529 AGCGGCAGCATCAACGCCTGGAGCACCAGGAGCCCTTCAGCTGGATCAAGGTGGACCTGCTGGCCCCCATG  
 1171▶ SerGlySerIleAsnAlaTrpSerThrLysGluProPheSerTrpIleLysValAspLeuLeuAlaProMet  
 3601 ATCATCCACGGCATCAAGACCCAGGGCGCCCGCCAGAAGTTCAGCAGCCTGTACATCAGCCAGTTCATCATC  
 1195▶ IleIleHisGlyIleLysThrGlnGlyAlaArgGlnLysPheSerSerLeuTyrIleSerGlnPheIleIle  
 3673 ATGTACAGCCTGGACGGCAAGAAGTGGCAGACCTACCGCGGCAACAGCACCGGCACCCTGATGGTGTCTTTC  
 1219▶ MetTyrSerLeuAspGlyLysLysTrpGlnThrTyrArgGlyAsnSerThrGlyThrLeuMetValPhePhe  
 (SmaI/EcoRV)

3745 GGCAACGTGGACAGCAGCGGCATCAAGCACAACATCTTCAACCCCCCATCATCGCCCGCTACATCCGCCTG  
 1243▶ GlyAsnValAspSerSerGlyIleLysHisAsnIlePheAsnProProIleIleAlaArgTyrIleArgLeu  
 3817 CACCCACCCACTACAGCATCCGCAGCACCCCTGCGCATGGAGCTGATGGGCTGCGACCTGAACAGCTGCAGC  
 1267▶ HisProThrHisTyrSerIleArgSerThrLeuArgMetGluLeuMetGlyCysAspLeuAsnSerCysSer  
 3889 ATGCCCTGGGCATGGAGAGCAAGGCCATCAGCGACGCCAGATCACCGCCAGCAGCTACTTCACCAACATG  
 1291▶ MetProLeuGlyMetGluSerLysAlaIleSerAspAlaGlnIleThrAlaSerSerTyrPheThrAsnMet  
 3961 TTCGCCACCTGGAGCCCCAGCAAGGCCCGCCTGCACCTGCAGGGCCGAGCAACGCCTGGCGCCCCCAGGTG  
 1315▶ PheAlaThrTrpSerProSerLysAlaArgLeuHisLeuGlnGlyArgSerAsnAlaTrpArgProGlnVal  
 BstEII

4033 AACAAACCCCAAGGAGTGGCTGCAGGTGGACTTCCAGAAGACCATGAAGGTGACCGGCGTGACCACCCAGGGC  
 1339▶ AsnAsnProLysGluTrpLeuGlnValAspPheGlnLysThrMetLysValThrGlyValThrThrGlnGly  
 4105 GTGAAGAGCCTGCTGACCAGCATGTACGTGAAGGAGTTCCTGATCAGCAGCAGCCAGGACGGCCACCACTGG  
 1363▶ ValLysSerLeuLeuThrSerMetTyrValLysGluPheLeuIleSerSerSerGlnAspGlyHisGlnTrp  
 4177 ACCCTGTTCTTCCAGAACGGCAAGGTGAAGGTGTTCCAGGGCAACCAGGACAGCTTCACCCCGTGGTGAAC  
 1387▶ ThrLeuPhePheGlnAsnGlyLysValLysValPheGlnGlyAsnGlnAspSerPheThrProValValAsn  
 4249 AGCCTGGACCCCCCTGCTGACCCGCTACCTGCGCATCCACCCCGAGAGCTGGGTGCACCAGATCGCCCTG  
 1411▶ SerLeuAspProProLeuLeuThrArgTyrLeuArgIleHisProGlnSerTrpValHisGlnIleAlaLeu  
 SmaI HindIII

4321 CGCATGGAGGTGCTGGGCTGCGAGGCCAGGACCTGTACTAGCTGCCCGGGCTACAAGCTTTAC  
 1435▶ ArgMetGluValLeuGlyCysGluAlaGlnAspLeuTyr...

FIG. 9 (3 of 3)



03407 Fig. 10 03407 03407 03407 03407

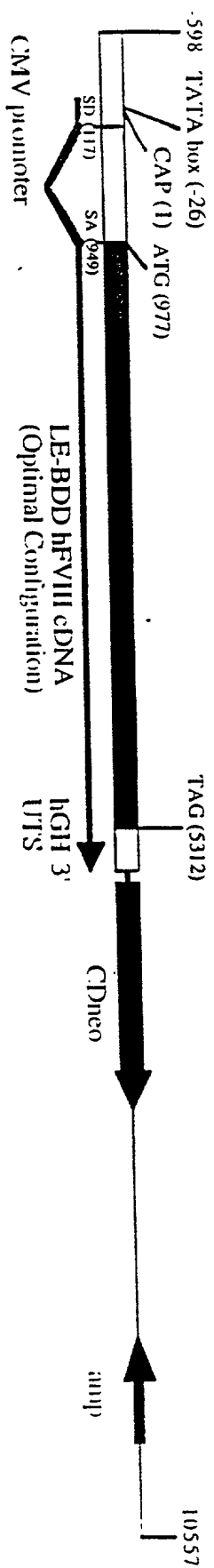
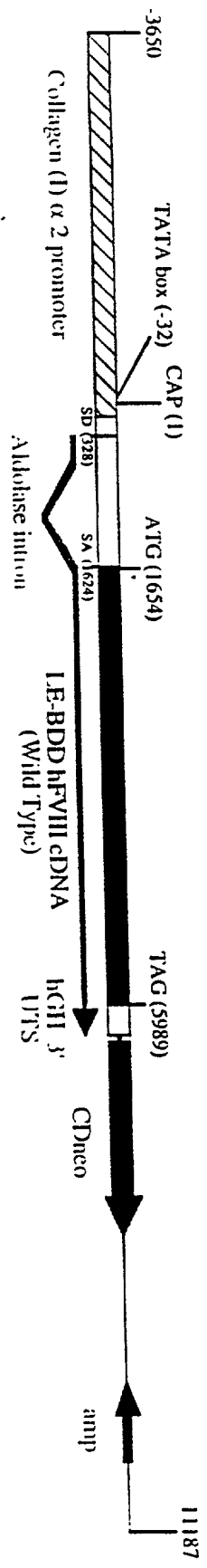


FIG. 11

00407606.000000





**FIG. 12**



# SEQUENCE LISTING

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Cys Leu Leu Arg Phe Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly	
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Ala Val Glu Leu Ser Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu	
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Pro Val Asp Ala Arg Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe	
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aac acc agc gtg gtg tac aag aag acc ctg ttc gtg gag ttc acc gac	243
Asn Thr Ser Val Val Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp	
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cac ctg ttc aac atc gcc aag ccc cgc ccc ccc tgg atg gcc ctg ctg	291
His Leu Phe Asn Ile Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu	
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ggc ccc acc atc cag gcc gag gtg tac gac acc gtg gtg atc acc ctg	339
Gly Pro Thr Ile Gln Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu	
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aag aac atg gcc agc cac ccc gtg agc ctg cac gcc gtg gcc gtg agc	387
Lys Asn Met Ala Ser His Pro Val Ser Leu His Ala Val Gly Val Ser	
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tac tgg aag gcc agc gag gcc gcc gag tac gac gac cag acc agc cag	435
Tyr Trp Lys Ala Ser Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln	
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Arg Glu Lys Glu Asp Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr	
140 145 150 155	
gtg tgg cag gtg ctg aag gag aac ggc ccc atg gcc agc gac ccc ctg	531
Val Trp Gln Val Leu Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu	
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tgc ctg acc tac agc tac ctg agc cac gtg gac ctg gtg aag gac ctg	579
Cys Leu Thr Tyr Ser Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu	
175 180 185	
aac agc ggc ctg atc ggc gcc ctg ctg gtg tgc cgc gag ggc agc ctg	627
Asn Ser Gly Leu Ile Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu	
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gcc aag gag aag acc cag acc ctg cac aag ttc atc ctg ctg ttc gcc	675
Ala Lys Glu Lys Thr Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala	
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gtg ttc gac gag ggc aag agc tgg cac agc gag acc aag aac agc ctg	723
Val Phe Asp Glu Gly Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu	
220 225 230 235	
atg cag gac cgc gac gcc gcc agc gcc cgc gcc tgg ccc aag atg cac	771
Met Gln Asp Arg Asp Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His	
240 245 250	
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Thr Val Asn Gly Tyr Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys	
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cac cgc aag agc gtg tac tgg cac gtg atc ggc atg ggc acc acc ccc	867
His Arg Lys Ser Val Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro	
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Glu Val His Ser Ile Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn	
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cac cgc cag gcc agc ctg gag atc agc ccc atc acc ttc ctg acc gcc	963
His Arg Gln Ala Ser Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala	
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Gln Thr Leu Leu Met Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile	
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Ser Ser His Gln His Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser	
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Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu	
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gac tac gac gac gac ctg acc gac agc gag atg gac gtg gtg cgc ttc	1155
Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe	
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Asp Asp Asp Asn Ser Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys	
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gtg ggc gac acc ctg ctg atc atc ttc aag aac cag gcc agc cgc ccc Val Gly Asp Thr Leu Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro 480 485 490	1491
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gag gtg gcc tac tgg tac atc ctg agc atc ggc gcc cag acc gac ttc Glu Val Ala Tyr Trp Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe 655 660 665	2019
ctg agc gtg ttc ttc agc ggc tac acc ttc aag cac aag atg gtg tac Leu Ser Val Phe Phe Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr 670 675 680	2067
gag gac acc ctg acc ctg ttc ccc ttc agc ggc gag acc gtg ttc atg Glu Asp Thr Leu Thr Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met 685 690 695	2115
agc atg gag aac ccc ggc ctg tgg atc ctg ggc tgc cac aac agc gac Ser Met Glu Asn Pro Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp 700 705 710 715	2163
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atc cgc gcc gag gtg gag gac aac atc atg gtg acc ttc cgc aac cag Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln 880 885 890	2691
gcc agc cgc ccc tac agc ttc tac agc agc ctg atc agc tac gag gag Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu 895 900 905	2739

gac cag cgc cag ggc gcc gag ccc cgc aag aac ttc gtg aag ccc aac Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys Pro Asn 910 915 920	2787
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cac acc aac acc ctg aac ccc gcc cac ggc cgc cag gtg acc gtg cag His Thr Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr Val Gln 975 980 985	2979
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cgc atc cgc tgg tac ctg ctg agc atg ggc agc aac gag aac atc cac Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn Ile His 1055 1060 1065	3219
agc atc cac ttc agc ggc cac gtg ttc acc gtg cgc aag aag gag gag Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys Glu Glu 1070 1075 1080	3267
tac aag atg gcc ctg tac aac ctg tac ccc ggc gtg ttc gag acc gtg Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu Thr Val 1085 1090 1095	3315
gag atg ctg ccc agc aag gcc ggc atc tgg cgc gtg gag tgc ctg atc Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys Leu Ile 1100 1105 1110 1115	3363
ggc gag cac ctg cac gcc ggc atg agc acc ctg ttc ctg gtg tac agc Gly Glu His Leu His Ala Gly Met Ser Thr Leu Phe Leu Val Tyr Ser 1120 1125 1130	3411
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gcc cgc ctg cac tac agc ggc agc atc aac gcc tgg agc acc aag gag Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr Lys Glu 1165 1170 1175	3555
ccc ttc agc tgg atc aag gtg gac ctg ctg gcc ccc atg atc atc cac Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile Ile His 1180 1185 1190 1195	3603
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cac ctg cag gcc cgc agc aac gcc tgg cgc ccc cag gtg aac aac ccc His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val Asn Asn Pro 1325 1330 1335	4035
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acc ccc gtg gtg aac agc ctg gac ccc ccc ctg ctg acc cgc tac ctg Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu Thr Arg Tyr Leu 1405 1410 1415	4275



cgc atc cac ccc cag agc tgg gtg cac cag atc gcc ctg cgc atg gag 4323  
 Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg Met Glu  
 1420 1425 1430 1435

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ttt 4376

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 35 40 45  
 Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val  
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 Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile  
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 Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln  
 85 90 95  
 Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser  
 100 105 110  
 His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser  
 115 120 125  
 Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp  
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 Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu  
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 Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser  
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 Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile  
 180 185 190  
 Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr  
 195 200 205  
 Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly  
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 Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp  
 225 230 235 240  
 Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr  
 245 250 255  
 Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val  
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 Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile  
 275 280 285  
 Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser  
 290 295 300  
 Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met  
 305 310 315 320  
 Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His  
 325 330 335  
 Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro  
 340 345 350

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Gln	Leu	Arg	Met	Lys	Asn	Asn	Glu	Glu	Ala	Glu	Asp	Tyr	Asp	Asp	Asp	
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Leu	Thr	Asp	Ser	Glu	Met	Asp	Val	Val	Arg	Phe	Asp	Asp	Asp	Asn	Ser	
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Pro	Ser	Phe	Ile	Gln	Ile	Arg	Ser	Val	Ala	Lys	Lys	His	Pro	Lys	Thr	
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Trp	Val	His	Tyr	Ile	Ala	Ala	Glu	Glu	Glu	Asp	Trp	Asp	Tyr	Ala	Pro	
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Leu	Val	Leu	Ala	Pro	Asp	Asp	Arg	Ser	Tyr	Lys	Ser	Gln	Tyr	Leu	Asn	
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Asn	Gly	Pro	Gln	Arg	Ile	Gly	Arg	Lys	Tyr	Lys	Lys	Val	Arg	Phe	Met	
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Ala	Tyr	Thr	Asp	Glu	Thr	Phe	Lys	Thr	Arg	Glu	Ala	Ile	Gln	His	Glu	
		450				455					460					
Ser	Gly	Ile	Leu	Gly	Pro	Leu	Leu	Tyr	Gly	Glu	Val	Gly	Asp	Thr	Leu	
		465			470					475					480	
Leu	Ile	Ile	Phe	Lys	Asn	Gln	Ala	Ser	Arg	Pro	Tyr	Asn	Ile	Tyr	Pro	
				485					490					495		
His	Gly	Ile	Thr	Asp	Val	Arg	Pro	Leu	Tyr	Ser	Arg	Arg	Leu	Pro	Lys	
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Gly	Val	Lys	His	Leu	Lys	Asp	Phe	Pro	Ile	Leu	Pro	Gly	Glu	Ile	Phe	
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Lys	Tyr	Lys	Trp	Thr	Val	Thr	Val	Glu	Asp	Gly	Pro	Thr	Lys	Ser	Asp	
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Pro	Arg	Cys	Leu	Thr	Arg	Tyr	Tyr	Ser	Ser	Phe	Val	Asn	Met	Glu	Arg	
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Asp	Leu	Ala	Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Ile	Cys	Tyr	Lys	Glu	
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Ile	Leu	Phe	Ser	Val	Phe	Asp	Glu	Asn	Arg	Ser	Trp	Tyr	Leu	Thr	Glu	
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Asn	Ile	Gln	Arg	Phe	Leu	Pro	Asn	Pro	Ala	Gly	Val	Gln	Leu	Glu	Asp	
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Pro	Glu	Phe	Gln	Ala	Ser	Asn	Ile	Met	His	Ser	Ile	Asn	Gly	Tyr	Val	
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Phe	Asp	Ser	Leu	Gln	Leu	Ser	Val	Cys	Leu	His	Glu	Val	Ala	Tyr	Trp	
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Tyr	Ile	Leu	Ser	Ile	Gly	Ala	Gln	Thr	Asp	Phe	Leu	Ser	Val	Phe	Phe	
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Ser	Gly	Tyr	Thr	Phe	Lys	His	Lys	Met	Val	Tyr	Glu	Asp	Thr	Leu	Thr	
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Leu	Phe	Pro	Phe	Ser	Gly	Glu	Thr	Val	Phe	Met	Ser	Met	Glu	Asn	Pro	
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Gly	Leu	Trp	Ile	Leu	Gly	Cys	His	Asn	Ser	Asp	Phe	Arg	Asn	Arg	Gly	
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				725					730					735		
Tyr	Tyr	Glu	Asp	Ser	Tyr	Glu	Asp	Ile	Ser	Ala	Tyr	Leu	Leu	Ser	Lys	
			740					745					750			
Asn	Asn	Ala	Ile	Glu	Pro	Arg	Leu	Glu	Glu	Ile	Thr	Arg	Thr	Thr	Leu	
		755					760					765				
Gln	Ser	Asp	Gln	Glu	Glu	Ile	Asp	Tyr	Asp	Asp	Thr	Ile	Ser	Val	Glu	
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Met	Lys	Lys	Glu	Asp	Phe	Asp	Ile	Tyr	Asp	Glu	Asp	Glu	Asn	Gln	Ser	
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Pro	Arg	Ser	Phe	Gln	Lys	Lys	Thr	Arg	His	Tyr	Phe	Ile	Ala	Ala	Val	
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Glu	Arg	Leu	Trp	Asp	Tyr	Gly	Met	Ser	Ser	Ser	Pro	His	Val	Leu	Arg	
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Asn	Arg	Ala	Gln	Ser	Gly	Ser	Val	Pro	Gln	Phe	Lys	Lys	Val	Val	Phe	
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Gln	Glu	Phe	Thr	Asp	Gly	Ser	Phe	Thr	Gln	Pro	Leu	Tyr	Arg	Gly	Glu	
						855					860					

Ser Ser Gln Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys  
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 Val Lys Val Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn  
 1395 1400 1405  
 Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln  
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 <223> Modified HUN2AN Factor VIII

<400> 3  
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 Met Gln Ile Glu Leu Ser Thr Cys Phe Phe Leu  
 1 5 10  
 tgc ctg ctg cgc ttc tgc ttc agc gcc acc cgc cgc tac tac ctg ggc 99  
 Cys Leu Leu Arg Phe Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly  
 15 20 25  
 gcc gtg gag ctg agc tgg gac tac atg cag agc gac ctg ggc gag ctg 147  
 Ala Val Glu Leu Ser Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu  
 30 35 40  
 ccc gtg gac gcc cgc ttc ccc ccc cgc gtg ccc aag agc ttc ccc ttc 195  
 Pro Val Asp Ala Arg Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe  
 45 50 55  
 aac acc agc gtg gtg tac aag aag acc ctg ttc gtg gag ttc acc gac 243  
 Asn Thr Ser Val Val Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp  
 60 65 70 75  
 cac ctg ttc aac atc gcc aag ccc cgc ccc ccc tgg atg ggc ctg ctg 291  
 His Leu Phe Asn Ile Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu  
 80 85 90  
 ggc ccc acc atc cag gcc gag gtg tac gac acc gtg gtg atc acc ctg 339  
 Gly Pro Thr Ile Gln Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu  
 95 100 105  
 aag aac atg gcc agc cac ccc gtg agc ctg cac gcc gtg ggc gtg agc 387  
 Lys Asn Met Ala Ser His Pro Val Ser Leu His Ala Val Gly Val Ser  
 110 115 120  
 tac tgg aag gcc agc gag ggc gcc gag tac gac gac cag acc agc cag 435  
 Tyr Trp Lys Ala Ser Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln  
 125 130 135  
 cgc gag aag gag gac gac aag gtg ttc ccc gcc gcc agc cac acc tac 483  
 Arg Glu Lys Glu Asp Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr  
 140 145 150 155

663660" 52323462

gtg tgg cag gtg ctg aag gag aac ggc ccc atg gcc agc gac ccc ctg Val Trp Gln Val Leu Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu 160 165 170	531
tgc ctg acc tac agc tac ctg agc cac gtg gac ctg gtg aag gac ctg Cys Leu Thr Tyr Ser Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu 175 180 185	579
aac agc ggc ctg atc ggc gcc ctg ctg gtg tgc cgc gag ggc agc ctg Asn Ser Gly Leu Ile Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu 190 195 200	627
gcc aag gag aag acc cag acc ctg cac aag ttc atc ctg ctg ttc gcc Ala Lys Glu Lys Thr Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala 205 210 215	675
gtg ttc gac gag ggc aag agc tgg cac agc gag acc aag aac agc ctg Val Phe Asp Glu Gly Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu 220 225 230 235	723
atg cag gac cgc gac gcc gcc agc gcc cgc gcc tgg ccc aag atg cac Met Gln Asp Arg Asp Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His 240 245 250	771
acc gtg aac ggc tac gtg aac cgc agc ctg ccc ggc ctg atc ggc tgc Thr Val Asn Gly Tyr Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys 255 260 265	819
cac cgc aag agc gtg tac tgg cac gtg atc ggc atg ggc acc acc ccc His Arg Lys Ser Val Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro 270 275 280	867
gag gtg cac agc atc ttc ctg gag ggc cac acc ttc ctg gtg cgc aac Glu Val His Ser Ile Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn 285 290 295	915
cac cgc cag gcc agc ctg gag atc agc ccc atc acc ttc ctg acc gcc His Arg Gln Ala Ser Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala 300 305 310 315	963
cag acc ctg ctg atg gac ctg ggc cag ttc ctg ctg ttc tgc cac atc Gln Thr Leu Leu Met Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile 320 325 330	1011
agc agc cac cag cac gac ggc atg gag gcc tac gtg aag gtg gac agc Ser Ser His Gln His Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser 335 340 345	1059
tgc ccc gag gag ccc cag ctg cgc atg aag aac aac gag gag gcc gag Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu 350 355 360	1107
gac tac gac gac gac ctg acc gac agc gag atg gac gtg gtg cgc ttc Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe 365 370 375	1155
gac gac gac aac agc ccc agc ttc atc cag atc cgc agc gtg gcc aag Asp Asp Asp Asn Ser Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys 380 385 390 395	1203
aag cac ccc aag acc tgg gtg cac tac atc gcc gcc gag gag gag gac Lys His Pro Lys Thr Trp Val His Tyr Ile Ala Ala Glu Glu Glu Asp 400 405 410	1251

tgg gac tac gcc ccc ctg gtg ctg gcc ccc gac gac cgc agc tac aag Trp Asp Tyr Ala Pro Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys 415 420 425	1299
agc cag tac ctg aac aac ggc ccc cag cgc atc ggc cgc aag tac aag Ser Gln Tyr Leu Asn Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys 430 435 440	1347
aag gtg cgc ttc atg gcc tac acc gac gag acc ttc aag acc cgc gag Lys Val Arg Phe Met Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu 445 450 455	1395
gcc atc cag cac gag agc ggc atc ctg ggc ccc ctg ctg tac ggc gag Ala Ile Gln His Glu Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu 460 465 470 475	1443
gtg ggc gac acc ctg ctg atc atc ttc aag aac cag gcc agc cgc ccc Val Gly Asp Thr Leu Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro 480 485 490	1491
tac aac atc tac ccc cac ggc atc acc gac gtg cgc ccc ctg tac agc Tyr Asn Ile Tyr Pro His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser 495 500 505	1539
cgc cgc ctg ccc aag ggc gtg aag cac ctg aag gac ttc ccc atc ctg Arg Arg Leu Pro Lys Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu 510 515 520	1587
ccc ggc gag atc ttc aag tac aag tgg acc gtg acc gtg gag gac ggc Pro Gly Glu Ile Phe Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly 525 530 535	1635
ccc acc aag agc gac ccc cgc tgc ctg acc cgc tac tac agc agc ttc Pro Thr Lys Ser Asp Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe 540 545 550 555	1683
gtg aac atg gag cgc gac ctg gcc agc ggc ctg atc ggc ccc ctg ctg Val Asn Met Glu Arg Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu 560 565 570	1731
atc tgc tac aag gag agc gtg gac cag cgc ggc aac cag atc atg agc Ile Cys Tyr Lys Glu Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser 575 580 585	1779
gac aag cgc aac gtg atc ctg ttc agc gtg ttc gac gag aac cgc agc Asp Lys Arg Asn Val Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser 590 595 600	1827
tgg tac ctg acc gag aac atc cag cgc ttc ctg ccc aac ccc gcc ggc Trp Tyr Leu Thr Glu Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly 605 610 615	1875
gtg cag ctg gag gac ccc gag ttc cag gcc agc aac atc atg cac agc Val Gln Leu Glu Asp Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser 620 625 630 635	1923
atc aac ggc tac gtg ttc gac agc ctg cag ctg agc gtg tgc ctg cac Ile Asn Gly Tyr Val Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His 640 645 650	1971
gag gtg gcc tac tgg tac atc ctg agc atc ggc gcc cag acc gac ttc Glu Val Ala Tyr Trp Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe 655 660 665	2019

ctg agc gtg ttc ttc agc ggc tac acc ttc aag cac aag atg gtg tac Leu Ser Val Phe Phe Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr 670 675 680	2067
gag gac acc ctg acc ctg ttc ccc ttc agc ggc gag acc gtg ttc atg Glu Asp Thr Leu Thr Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met 685 690 695	2115
agc atg gag aac ccc ggc ctg tgg atc ctg ggc tgc cac aac agc gac Ser Met Glu Asn Pro Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp 700 705 710 715	2163
ttc cgc aac cgc ggc atg acc gcc ctg ctg aag gtg agc agc tgc gac Phe Arg Asn Arg Gly Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp 720 725 730	2211
aag aac acc ggc gac tac tac gag gac agc tac gag gac atc agc gcc Lys Asn Thr Gly Asp Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala 735 740 745	2259
tac ctg ctg agc aag aac aac gcc atc gag ccc cgc agg cgc agg cgc Tyr Leu Leu Ser Lys Asn Asn Ala Ile Glu Pro Arg Arg Arg Arg Arg 750 755 760	2307
gag atc acc cgc acc acc ctg cag agc gac cag gag gag atc gac tac Glu Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Ile Asp Tyr 765 770 775	2355
gac gac acc atc agc gtg gag atg aag aag gag gac ttc gac atc tac Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr 780 785 790 795	2403
gac gag gac gag aac cag agc ccc cgc agc ttc cag aag aag acc cgc Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg 800 805 810	2451
cac tac ttc atc gcc gcc gtg gag cgc ctg tgg gac tac ggc atg agc His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser 815 820 825	2499
agc agc ccc cac gtg ctg cgc aac cgc gcc cag agc ggc agc gtg ccc Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro 830 835 840	2547
cag ttc aag aag gtg gtg ttc cag gag ttc acc gac ggc agc ttc acc Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe Thr 845 850 855	2595
cag ccc ctg tac cgc ggc gag ctg aac gag cac ctg ggc ctg ctg ggc Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu Leu Gly 860 865 870 875	2643
ccc tac atc cgc gcc gag gtg gag gac aac atc atg gtg acc ttc cgc Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg 880 885 890	2691
aac cag gcc agc cgc ccc tac agc ttc tac agc agc ctg atc agc tac Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser Leu Ile Ser Tyr 895 900 905	2739
gag gag gac cag cgc cag ggc gcc gag ccc cgc aag aac ttc gtg aag Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys 910 915 920	2787

ccc aac gag acc aag acc tac ttc tgg aag gtg cag cac cac atg gcc Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val Gln His His Met Ala 925 930 935	2835
ccc acc aag gac gag ttc gac tgc aag gcc tgg gcc tac ttc agc gac Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp 940 945 950 955	2883
gtg gac ctg gag aag gac gtg cac agc ggc ctg atc ggc ccc ctg ctg Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu 960 965 970	2931
gtg tgc cac acc aac acc ctg aac ccc gcc cac ggc cgc cag gtg acc Val Cys His Thr Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr 975 980 985	2979
gtg cag gag ttc gcc ctg ttc ttc acc atc ttc gac gag acc aag agc Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser 990 995 1000	3027
tgg tac ttc acc gag aac atg gag cgc aac tgc cgc gcc ccc tgc aac Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn 1005 1010 1015	3075
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atc aac ggc tac atc atg gac acc ctg ccc ggc ctg gtg atg gcc cag Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala Gln 1040 1045 1050	3171
gac cag cgc atc cgc tgg tac ctg ctg agc atg ggc agc aac gag aac Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn 1055 1060 1065	3219
atc cac agc atc cac ttc agc ggc cac gtg ttc acc gtg cgc aag aag Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys 1070 1075 1080	3267
gag gag tac aag atg gcc ctg tac aac ctg tac ccc ggc gtg ttc gag Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu 1085 1090 1095	3315
acc gtg gag atg ctg ccc agc aag gcc ggc atc tgg cgc gtg gag tgc Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys 1100 1105 1110 1115	3363
ctg atc ggc gag cac ctg cac gcc ggc atg agc acc ctg ttc ctg gtg Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu Phe Leu Val 1120 1125 1130	3411
tac agc aac aag tgc cag acc ccc ctg ggc atg gcc agc ggc cac atc Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His Ile 1135 1140 1145	3459
cgc gac ttc cag atc acc gcc agc ggc cag tac ggc cag tgg gcc ccc Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp Ala Pro 1150 1155 1160	3507
aag ctg gcc cgc ctg cac tac agc ggc agc atc aac gcc tgg agc acc Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr 1165 1170 1175	3555

aag gag ccc ttc agc tgg atc aag gtg gac ctg ctg gcc ccc atg atc Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile 1180 1185 1190 1195	3603
atc cac ggc atc aag acc cag ggc gcc cgc cag aag ttc agc agc ctg Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu 1200 1205 1210	3651
tac atc agc cag ttc atc atc atg tac agc ctg gac ggc aag aag tgg Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp 1215 1220 1225	3699
cag acc tac cgc ggc aac agc acc ggc acc ctg atg gtg ttc ttc ggc Gln Thr Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val Phe Phe Gly 1230 1235 1240	3747
aac gtg gac agc agc ggc atc aag cac aac atc ttc aac ccc ccc atc Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile 1245 1250 1255	3795
atc gcc cgc tac atc cgc ctg cac ccc acc cac tac agc atc cgc agc Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser 1260 1265 1270 1275	3843
acc ctg cgc atg gag ctg atg ggc tgc gac ctg aac agc tgc agc atg Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met 1280 1285 1290	3891
ccc ctg ggc atg gag agc aag gcc atc agc gac gcc cag atc acc gcc Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala 1295 1300 1305	3939
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cgc ctg cac ctg cag ggc cgc agc aac gcc tgg cgc ccc cag gtg aac Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val Asn 1325 1330 1335	4035
aac ccc aag gag tgg ctg cag gtg gac ttc cag aag acc atg aag gtg Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met Lys Val 1340 1345 1350 1355	4083
acc ggc gtg acc acc cag ggc gtg aag agc ctg ctg acc agc atg tac Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr Ser Met Tyr 1360 1365 1370	4131
gtg aag gag ttc ctg atc agc agc agc cag gac ggc cac cag tgg acc Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly His Gln Trp Thr 1375 1380 1385	4179
ctg ttc ttc cag aac ggc aag gtg aag gtg ttc cag ggc aac cag gac Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp 1390 1395 1400	4227
agc ttc acc ccc gtg gtg aac agc ctg gac ccc ccc ctg ctg acc cgc Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg 1405 1410 1415	4275
tac ctg cgc atc cac ccc cag agc tgg gtg cac cag atc gcc ctg cgc Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg 1420 1425 1430 1435	4323



atg gag gtg ctg ggc tgc gag gcc cag gac ctg tac tagctgccccg 4381  
Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr  
1440 1445

ggctacaagc tttaac 4396

<210> 4  
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<212> PRT  
<213> Artificial Sequence

<220>  
<221> Artificial Sequence  
<223> Modified HUN2AN Factor VIII

<400> 4  
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Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser  
20 25 30  
Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg  
35 40 45  
Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val  
50 55 60  
Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile  
65 70 75 80  
Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln  
85 90 95  
Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser  
100 105 110  
His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser  
115 120 125  
Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp  
130 135 140  
Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu  
145 150 155 160  
Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser  
165 170 175  
Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile  
180 185 190  
Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr  
195 200 205  
Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly  
210 215 220  
Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp  
225 230 235 240  
Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr  
245 250 255  
Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val  
260 265 270  
Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile  
275 280 285  
Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser  
290 295 300  
Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met  
305 310 315 320  
Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His  
325 330 335  
Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro  
340 345 350  
Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp  
355 360 365

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Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser  
 370 375 380  
 Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr  
 385 390 395 400  
 Trp Val His Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro  
 405 410 415  
 Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn  
 420 425 430  
 Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met  
 435 440 445  
 Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu  
 450 455 460  
 Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu  
 465 470 475 480  
 Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro  
 485 490 495  
 His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys  
 500 505 510  
 Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe  
 515 520 525  
 Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp  
 530 535 540  
 Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg  
 545 550 555 560  
 Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu  
 565 570 575  
 Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val  
 580 585 590  
 Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu  
 595 600 605  
 Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp  
 610 615 620  
 Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val  
 625 630 635 640  
 Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp  
 645 650 655  
 Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe  
 660 665 670  
 Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr  
 675 680 685  
 Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro  
 690 695 700  
 Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly  
 705 710 715 720  
 Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp  
 725 730 735  
 Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys  
 740 745 750  
 Asn Asn Ala Ile Glu Pro Arg Arg Arg Arg Arg Glu Ile Thr Arg Thr  
 755 760 765  
 Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser  
 770 775 780  
 Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn  
 785 790 795 800  
 Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala  
 805 810 815  
 Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val  
 820 825 830  
 Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val  
 835 840 845  
 Val Phe Gln Glu Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg  
 850 855 860  
 Gly Glu Leu Asn Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala  
 865 870 875 880

Glu	Val	Glu	Asp	Asn	Ile	Met	Val	Thr	Phe	Arg	Asn	Gln	Ala	Ser	Arg	885	890	895
Pro	Tyr	Ser	Phe	Tyr	Ser	Ser	Leu	Ile	Ser	Tyr	Glu	Glu	Asp	Gln	Arg	900	905	910
Gln	Gly	Ala	Glu	Pro	Arg	Lys	Asn	Phe	Val	Lys	Pro	Asn	Glu	Thr	Lys	915	920	925
Thr	Tyr	Phe	Trp	Lys	Val	Gln	His	His	Met	Ala	Pro	Thr	Lys	Asp	Glu	930	935	940
Phe	Asp	Cys	Lys	Ala	Trp	Ala	Tyr	Phe	Ser	Asp	Val	Asp	Leu	Glu	Lys	945	950	955
Asp	Val	His	Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Val	Cys	His	Thr	Asn	965	970	975
Thr	Leu	Asn	Pro	Ala	His	Gly	Arg	Gln	Val	Thr	Val	Gln	Glu	Phe	Ala	980	985	990
Leu	Phe	Phe	Thr	Ile	Phe	Asp	Glu	Thr	Lys	Ser	Trp	Tyr	Phe	Thr	Glu	995	1000	1005
Asn	Met	Glu	Arg	Asn	Cys	Arg	Ala	Pro	Cys	Asn	Ile	Gln	Met	Glu	Asp	1010	1015	1020
Pro	Thr	Phe	Lys	Glu	Asn	Tyr	Arg	Phe	His	Ala	Ile	Asn	Gly	Tyr	Ile	1025	1030	1035
Met	Asp	Thr	Leu	Pro	Gly	Leu	Val	Met	Ala	Gln	Asp	Gln	Arg	Ile	Arg	1045	1050	1055
Trp	Tyr	Leu	Leu	Ser	Met	Gly	Ser	Asn	Glu	Asn	Ile	His	Ser	Ile	His	1060	1065	1070
Phe	Ser	Gly	His	Val	Phe	Thr	Val	Arg	Lys	Lys	Glu	Glu	Tyr	Lys	Met	1075	1080	1085
Ala	Leu	Tyr	Asn	Leu	Tyr	Pro	Gly	Val	Phe	Glu	Thr	Val	Glu	Met	Leu	1090	1095	1100
Pro	Ser	Lys	Ala	Gly	Ile	Trp	Arg	Val	Glu	Cys	Leu	Ile	Gly	Glu	His	1105	1110	1115
Leu	His	Ala	Gly	Met	Ser	Thr	Leu	Phe	Leu	Val	Tyr	Ser	Asn	Lys	Cys	1125	1130	1135
Gln	Thr	Pro	Leu	Gly	Met	Ala	Ser	Gly	His	Ile	Arg	Asp	Phe	Gln	Ile	1140	1145	1150
Thr	Ala	Ser	Gly	Gln	Tyr	Gly	Gln	Trp	Ala	Pro	Lys	Leu	Ala	Arg	Leu	1155	1160	1165
His	Tyr	Ser	Gly	Ser	Ile	Asn	Ala	Trp	Ser	Thr	Lys	Glu	Pro	Phe	Ser	1170	1175	1180
Trp	Ile	Lys	Val	Asp	Leu	Leu	Ala	Pro	Met	Ile	Ile	His	Gly	Ile	Lys	1185	1190	1195
Thr	Gln	Gly	Ala	Arg	Gln	Lys	Phe	Ser	Ser	Leu	Tyr	Ile	Ser	Gln	Phe	1205	1210	1215
Ile	Ile	Met	Tyr	Ser	Leu	Asp	Gly	Lys	Lys	Trp	Gln	Thr	Tyr	Arg	Gly	1220	1225	1230
Asn	Ser	Thr	Gly	Thr	Leu	Met	Val	Phe	Phe	Gly	Asn	Val	Asp	Ser	Ser	1235	1240	1245
Gly	Ile	Lys	His	Asn	Ile	Phe	Asn	Pro	Pro	Ile	Ile	Ala	Arg	Tyr	Ile	1250	1255	1260
Arg	Leu	His	Pro	Thr	His	Tyr	Ser	Ile	Arg	Ser	Thr	Leu	Arg	Met	Glu	1265	1270	1275
Leu	Met	Gly	Cys	Asp	Leu	Asn	Ser	Cys	Ser	Met	Pro	Leu	Gly	Met	Glu	1285	1290	1295
Ser	Lys	Ala	Ile	Ser	Asp	Ala	Gln	Ile	Thr	Ala	Ser	Ser	Tyr	Phe	Thr	1300	1305	1310
Asn	Met	Phe	Ala	Thr	Trp	Ser	Pro	Ser	Lys	Ala	Arg	Leu	His	Leu	Gln	1315	1320	1325
Gly	Arg	Ser	Asn	Ala	Trp	Arg	Pro	Gln	Val	Asn	Asn	Pro	Lys	Glu	Trp	1330	1335	1340
Leu	Gln	Val	Asp	Phe	Gln	Lys	Thr	Met	Lys	Val	Thr	Gly	Val	Thr	Thr	1345	1350	1355
Gln	Gly	Val	Lys	Ser	Leu	Leu	Thr	Ser	Met	Tyr	Val	Lys	Glu	Phe	Leu	1365	1370	1375
Ile	Ser	Ser	Ser	Gln	Asp	Gly	His	Gln	Trp	Thr	Leu	Phe	Phe	Gln	Asn	1380	1385	1390



Leu	Asn	Glu	His	Leu	Gly	Leu	Leu	Gly	Pro	Tyr	Ile	Arg	Ala	Glu	Val
865					870					875					880
Glu	Asp	Asn	Ile	Met	Val	Thr	Phe	Arg	Asn	Gln	Ala	Ser	Arg	Pro	Tyr
				885					890					895	
Ser	Phe	Tyr	Ser	Ser	Leu	Ile	Ser	Tyr	Glu	Glu	Asp	Gln	Arg	Gln	Gly
			900					905					910		
Ala	Glu	Pro	Arg	Lys	Asn	Phe	Val	Lys	Pro	Asn	Glu	Thr	Lys	Thr	Tyr
		915					920						925		
Phe	Trp	Lys	Val	Gln	His	His	Met	Ala	Pro	Thr	Lys	Asp	Glu	Phe	Asp
930						935						940			
Cys	Lys	Ala	Trp	Ala	Tyr	Phe	Ser	Asp	Val	Asp	Leu	Glu	Lys	Asp	Val
945					950					955					960
His	Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Val	Cys	His	Thr	Asn	Thr	Leu
				965					970						975
Asn	Pro	Ala	His	Gly	Arg	Gln	Val	Thr	Val	Gln	Glu	Phe	Ala	Leu	Phe
			980					985						990	
Phe	Thr	Ile	Phe	Asp	Glu	Thr	Lys	Ser	Trp	Tyr	Phe	Thr	Glu	Asn	Met
		995					1000						1005		
Glu	Arg	Asn	Cys	Arg	Ala	Pro	Cys	Asn	Ile	Gln	Met	Glu	Asp	Pro	Thr
1010						1015						1020			
Phe	Lys	Glu	Asn	Tyr	Arg	Phe	His	Ala	Ile	Asn	Gly	Tyr	Ile	Met	Asp
1025					1030					1035					1040
Thr	Leu	Pro	Gly	Leu	Val	Met	Ala	Gln	Asp	Gln	Arg	Ile	Arg	Trp	Tyr
				1045					1050					1055	
Leu	Leu	Ser	Met	Gly	Ser	Asn	Glu	Asn	Ile	His	Ser	Ile	His	Phe	Ser
			1060					1065						1070	
Gly	His	Val	Phe	Thr	Val	Arg	Lys	Lys	Glu	Glu	Tyr	Lys	Met	Ala	Leu
		1075					1080						1085		
Tyr	Asn	Leu	Tyr	Pro	Gly	Val	Phe	Glu	Thr	Val	Glu	Met	Leu	Pro	Ser
		1090				1095						1100			
Lys	Ala	Gly	Ile	Trp	Arg	Val	Glu	Cys	Leu	Ile	Gly	Glu	His	Leu	His
1105					1110					1115					1120
Ala	Gly	Met	Ser	Thr	Leu	Phe	Leu	Val	Tyr	Ser	Asn	Lys	Cys	Gln	Thr
				1125					1130					1135	
Pro	Leu	Gly	Met	Ala	Ser	Gly	His	Ile	Arg	Asp	Phe	Gln	Ile	Thr	Ala
			1140					1145					1150		
Ser	Gly	Gln	Tyr	Gly	Gln	Trp	Ala	Pro	Lys	Leu	Ala	Arg	Leu	His	Tyr
		1155					1160						1165		
Ser	Gly	Ser	Ile	Asn	Ala	Trp	Ser	Thr	Lys	Glu	Pro	Phe	Ser	Trp	Ile
		1170				1175						1180			
Lys	Val	Asp	Leu	Leu	Ala	Pro	Met	Ile	Ile	His	Gly	Ile	Lys	Thr	Gln
1185					1190					1195					1200
Gly	Ala	Arg	Gln	Lys	Phe	Ser	Ser	Leu	Tyr	Ile	Ser	Gln	Phe	Ile	Ile
				1205					1210					1215	
Met	Tyr	Ser	Leu	Asp	Gly	Lys	Lys	Trp	Gln	Thr	Tyr	Arg	Gly	Asn	Ser
			1220					1225					1230		
Thr	Gly	Thr	Leu	Met	Val	Phe	Phe	Gly	Asn	Val	Asp	Ser	Ser	Gly	Ile
		1235					1240						1245		
Lys	His	Asn	Ile	Phe	Asn	Pro	Ile	Ile	Ala	Arg	Tyr	Ile	Arg	Leu	
		1250				1255					1260				
His	Pro	Thr	His	Tyr	Ser	Ile	Arg	Ser	Thr	Leu	Arg	Met	Glu	Leu	Met
1265					1270					1275					1280
Gly	Cys	Asp	Leu	Asn	Ser	Cys	Ser	Met	Pro	Leu	Gly	Met	Glu	Ser	Lys
				1285					1290					1295	
Ala	Ile	Ser	Asp	Ala	Gln	Ile	Thr	Ala	Ser	Ser	Tyr	Phe	Thr	Asn	Met
			1300					1305						1310	
Phe	Ala	Thr	Trp	Ser	Pro	Ser	Lys	Ala	Arg	Leu	His	Leu	Gln	Gly	Arg
		1315					1320						1325		
Ser	Asn	Ala	Trp	Arg	Pro	Gln	Val	Asn	Asn	Pro	Lys	Glu	Trp	Leu	Gln
		1330				1335					1340				
Val	Asp	Phe	Gln	Lys	Thr	Met	Lys	Val	Thr	Gly	Val	Thr	Thr	Gln	Gly
1345					1350					1355					1360
Val	Lys	Ser	Leu	Leu	Thr	Ser	Met	Tyr	Val	Lys	Glu	Phe	Leu	Ile	Ser
				1365					1370					1375	

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